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Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

Tel: 03-5840-8764, Fax: 03-5840-8765
E-mail: office@biosciencetrends.com
URL: www.biosciencetrends.com

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Japanese Red Cross Medical Center, Tokyo, Japan

Japanese Red Cross Medical Center moved to the current location at Hiroo, Tokyo in 1891. It is the leading hospital of the 92 Red Cross hospitals in Japan, serving the community in the areas of advanced cancer management, emergency medical service, and perinatal care, tying up with local clinics. This new hospital building started its operation at the beginning of January, 2010 with 573 general, and 117 ICU and other related special beds. The center looks to the future of medical care, but with the full understanding of responsibility Red Cross hospitals bear.

(Photo by Hiroaki Hayashi)

Review

Intimate partner violence in Sri Lanka

Achini C. Jayatilleke[1], Krishna C. Poudel[1,*], Junko Yasuoka[1], Achala U. Jayatilleke[1,2], Masamine Jimba[1]

[1] *Department of Community and Global Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[2] *Department of Public Health, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka.*

Summary **To describe the current situation of intimate partner violence (IPV) in Sri Lanka, and to propose possible interventions to prevent IPV, we performed a literature survey for articles and reports on IPV in Sri Lanka. Our results suggested that prevalence of IPV is high (40%) in Sri Lanka. Most of the IPV studies were conducted in health care institutions and missed IPV victims who had not attended a health care institution. A common belief in Sri Lanka, even among medical students and police officers is that IPV is a personal matter that outsiders should not intervene. The laws against IPV identify the physical and psychological IPV, but not the sexual IPV. To improve this situation of IPV in Sri Lanka, we recommend IPV education programs for medical students and police officers, community awareness programs on IPV, and amending the laws to identify sexual IPV. We also recommend well designed community based research on IPV.**

Keywords: Human rights, intimate partner violence, patterns, prevalence, Sri Lanka, women

1. Introduction

Intimate partner violence (IPV) is considered a global public health problem (*1*), however the burden of IPV falls most heavily on developing countries (*2*). To address such violence, several initiatives were taken at the international level. In 1979, the United Nations adopted "The Convention on the Elimination of All Forms of Discrimination against Women" (CEDAW). In the 1995 United Nations' Beijing World Conference on Women, 189 governments signed on to a platform for action to prevent violence against women including IPV (*3-5*). At the regional level, "The Jakarta Declaration for the Advancement of Women in Asia and the Pacific" was adopted in 1994. This declaration presented some specific goals, objectives, and actions to be taken by governments to address violence against women (*4*).

With these initiatives, IPV became a topic of discussion in many Asian countries. This discussion has been strengthened by some evidence on IPV. In Asia, there are data on IPV from India, Bangladesh, Thailand, and Cambodia because they have been included in multi-country studies on IPV conducted by international organizations (*1,2*). Those studies indicated that the prevalence of IPV varies between 18% in Cambodia and 40% in Bangladesh. In India, the prevalence of IPV is 19%, and in Thailand, 34%. However, Sri Lanka and other low-income Asian countries have not been included in such studies.

Sri Lanka was one of the first countries in South Asia to ratify the CEDAW in 1981. In 1995, Sri Lanka also subscribed to the Beijing platform for action to prevent violence against women (*3,4*), although little is known about the prevalence of IPV in the country (*4*). To date, no literature review has been undertaken about IPV in Sri Lanka. Therefore, we performed a review of the published literature on IPV in Sri Lanka over the past 28 years to describe the prevalence, patterns, contributing factors, the attitudes towards IPV, and IPV prevention activities in Sri Lanka. Using these literatures, we aimed to propose possible interventions to improve the current situation of IPV in Sri Lanka.

2. Data Sources

In April 2009, we performed this literature survey in the databases of MEDLINE®, PsycINFO®, and

**Address correspondence to:*
Dr. Krishna C. Poudel, Department of Community and Global Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
e-mail: krishna@m.u-tokyo.ac.jp

POPLINE® for articles on IPV in Sri Lanka. We used Medical Subject Headings (MeSH) key words, such as 'intimate partner violence', 'domestic violence', 'violence against women', 'abuse', and 'Sri Lanka'. We surveyed for articles written in English and published since January 1980. We also searched for documents on IPV in Sri Lanka published by international organizations, such as the United Nations and other non-governmental organizations (NGOs). We surveyed for publications written in English and published from 1990 onwards.

The search of the databases produced 91 articles. However, only eight articles contained information on IPV in Sri Lanka (*6-13*). The remaining 83 articles were excluded as they were related to child abuse, female domestic workers, female suicide, and the victimization of women in the armed conflicts of the northern and eastern provinces of Sri Lanka.

The survey for the publications by international organizations produced other eight articles. Of them, one was a research report (*14*), two were country reports by the United Nations (*5,15*), and five were articles by the NGOs working on violence against women in Sri Lanka (*3,4,16-19*).

From these 16 articles, we extracted the specific information on IPV, but not the other forms of gender-based violence against women such as violence by the in-laws, the community, and the work place violence. We described the probable prevalence, the patterns, the contributing factors, and the attitudes towards IPV in the country based on the combined data from these articles.

3. Prevalence and patterns of IPV in Sri Lanka

As shown in Table 1, the first research on IPV in Sri Lanka was published in 1982 (*6*). It involved the analysis of married or cohabiting women who had been repeatedly assaulted by their husbands or partners (n = 60). Participants were recruited between August 1978 and August 1981 in their medico-legal examinations at the medico-legal department of a leading General Hospital. Sixty-two percent of the participants were assaulted by their husbands/partners with weapons such as sticks, firewood, and kitchen knives. Among the remaining 38%, 18% were punched, 5% were kicked, and 15% were subjected to other forms of violence, such as burning, strangulation, and so forth.

In 2001, a cross-sectional study on wife beating was published (*7*). This study was conducted as a household survey in a rural primary health care area of Sri Lanka. The participants were married or cohabiting women between 18-49 years of age whom were randomly selected using eligible couple registers (n = 417). The results indicated that 30% of the women had been physically abused by their spouse at some point in their lives, and 22% had experienced physical abuse within the past year. Contusions were the most commonly reported injuries (65%). However, in contrast to the previous reference, this study reported that 88% of the women were assaulted manually, without using weapons.

In 2001, another study was conducted in an antenatal clinic setting with pregnant women between 15-49 years old (n = 1,200). The participants were selected by multi stage cluster randomized sampling method using the pregnant mothers' registers maintained by the public health midwives in a single district (*8*). In addition to investigating IPV, this study examined other forms of domestic violence experienced by pregnant women. Of the 1,200 participants, 4.7% reported physical abuse during their current pregnancy. Of these, 20% were physically abused at least once a week, and in more than 70% of the cases, the husband or the male partner perpetrated the violence.

A 2003 study was conducted with ever married women attending the outpatient department of a General Hospital (n = 242) (*9*). Of all the participants, 41% have indicated that they experienced one of physical, verbal, emotional, or sexual abuse from their husbands. Of them 27% had contracted injuries due to the violence. However, only two had disclosed the actual reason for their injuries.

In 2005, a study was conducted in the tsunami affected areas in Sri Lanka (*14*). In that study, the author had conducted in-depth, semi-structured interviews with the NGO officers involved in gender based violence prevention activities in that area. The interviews have revealed that IPV was common during the post tsunami period. For example, one woman has been constantly abused by her husband who blamed her for the loss of their children because she was with them when they were lost in the tsunami. Another woman has suffered major burns after being set on fire by her drunken husband.

4. Contributing factors for IPV in Sri Lanka

4.1. *Socio-demographic factors*

As indicated in Table 1, in 1982, the first study on IPV was published in Sri Lanka. This study reported that in 70% of the cases, wives had experienced IPV when their husbands were intoxicated (*6*). In 2001, the study on wife beating reported that wife beating was associated with an early age at marriage, low income, and large families (*7*). The study conducted in the post tsunami period reported that IPV was common during the post tsunami period due to the frustration and stress associated with communal living in refugee camps, feeling of loss and trauma, and men's increased alcohol consumption.

4.2. *Patriarchal attitudes*

In patriarchal Sri Lanka, the family expects wives

Table 1. Details of articles on intimate partner violence in Sri Lanka

Reference	Setting	Participants	Data collection	Main findings
Saravanapavanathan (1982)	Medico-legal Department, Teaching Hospital, Jaffna	Repeatedly (three or more times) assaulted, married/ cohabiting women (n = 60)	Interviewer administered questionnaire	Assaulted with weapons like sticks, fire wood (62%), punched (18%), kicked (5%), burning and strangulation (15%). Contracted bruises (60%), lacerations (22%), incised wounds (8%). Injuries in head (42%), upper limbs (37%), lower limbs (10%), other (16%).
Subramanium (2001)	Primary health care area, Kantale	Married or cohabiting women between 18-49 years (n = 417)	Focus group discussions and interviewer administered questionnaire	Lifetime prevalence of wife beating (30%), wife beating during past year (22%). Determinants of wife beating are early age of marriage for women (less than 18 years), low standard of living index (less than 3), alcohol consumption by husband. Commonest type of injury are contusions (65%). Commonest sites of injuries are head, face and neck (60%). Commonest type of assault is manual assaults (87.5%).
Moonasinghe (2004)	Antenatal clinic, Badulla district	Married or cohabiting pregnant women between 15-49 years (n = 1,200)	Interviewer administered Questionnaire	Prevalence of ever abuse (18.3%), current abuse (10.6%), abuse during pregnancy (4.7%), current sexual abuse (2.7%). Perpetrators of current physical abuse are husband (72.4%), mother-in-law (8.8%), others (18.8%). Perpetrators of current sexual abuse are husband/male partner (81.8%), father (9.1%), other (9.1%).
Kuruppuarachchi and Wijeratne (2005)	Out Patient Department, North Colombo Teaching Hospital, Ragama	Women attending Out Patient Department	Interviewer administered questionnaire	Prevalence of physical abuse (19%), psychological abuse (23%), sexual abuse (7%), at least one of physical, psychological and/or sexual abuse (41%).
Fisher (2005)	Tsunami affected areas in Sri Lanka	Key staff of NGOs known to be working on gender based violence initiatives	In-depth, semi-structured interviews	Intimate partner violence is widespread in Tsunami welfare camps. Contributing factors for IPV are increased alcohol consumption by husbands, stress, psychological trauma and lack of privacy.
Haj-Yahia (2007)	Faculty of Medicine, University of Colombo	1st, 2nd, 3rd and 4th year medical students (n = 476) First year-64%, Second year-4%, Third year-14%, Fourth year-18%	Self administered questionnaire	Medical students justify beating of a sexually unfaithful wife (33.4%), beating of a constantly disobeying wife (25.1%). Medical students believe women benefit from wife beating (24%), women can avoid being battered (63.1%), social services should help battered women (86.1%), husbands who beat their wives should be arrested by police (8.7%).
Mason (2008)	Toronto, Canada	Sri Lankan immigrant Tamil Women 18-24 years (n = 17) 25-64 years (n = 16) > 65 years (n = 18) Had counseling for IPV (n = 12)	Focus group interviews	Participants define IPV broadly as physical, sexual, emotional/psychological, and financial abuse. Forms of intolerable abuse are excessive suspicion, jealousy and anxiety about wives' fidelity. Marriages are commonly arranged by parents. In them, men commonly lie about their job and request higher dowries.

to uphold cultural values, and act in a manner that does not bring shame on the family (*4,12*). Marriage and motherhood are still social norms for Sri Lankan women. Social disapproval for separation or divorce makes it difficult for women to escape abusive marriages. This enduring nature of IPV is suggested by the low divorce rate in Sri Lanka despite the high prevalence of IPV (*4,18*).

A research letter published in 2008 present three case reports of Sri Lankan wives who experienced physical, psychological, and sexual IPV from their husbands (*13*). In all those cases, wives were well educated and had good socio-economic backgrounds. However, before sorting help to prevent IPV, they had been tolerating the violence for years due to the lack of the external support, social stigma, and their hesitancy to challenge the patriarchal norms.

In 2004, a study was conducted in Toronto, Canada with a group of immigrant Sri Lankan Tamil women above 18 years old (n = 63) (*11*). This study examined the Tamil women's perceptions of IPV. The participants were recruited using snowballing techniques, flyers, community organizations, and an article in a local Tamil newspaper.

The focus group interviews have revealed that immigrant Sri Lankan Tamil women define IPV broadly as physical, psychological/emotional, sexual and financial abuse. Excessive suspicion, jealousy, and anxiety about wives' fidelity were mentioned as forms of intolerable abuse. Even in Canada, Tamil women have acknowledged that divorce or separation tend to lower their status in the society. Further, dowry related abuse such as complains of insufficient dowries and unfulfilled dowry promises still exist in this community (*11*).

In 2006, a study was conducted with undergraduate medical students (50.6% male and 49.4% female) in a leading university in Sri Lanka (n = 476), who responded to an open announcement and participated voluntarily in the study (*10*). This study explored the medical students' attitudes towards wife beating. The results indicated that 33.4% of the students justified wife beating, and 63.1% stated that they believed women bear a proportionately larger responsibility for the violence perpetrated against them. A further 23.2% of the students reported that they believed that occasional violence by a husband towards his wife could help to maintain their marriage. In addition, the majority opposed divorce as a solution for wife abuse and disagreed with punishing violent husbands.

4.3. *Inadequate professional response for IPV*

The country reports by the World Organization Against Torture and PANOS Sri Lanka suggest that most police officers in Sri Lanka fail to respond to the complaints of IPV in a gender sensitive and effective manner (*4,16*). Although an average of 4,000 cases of domestic violence including IPV were reported to the police every month, nearly 80% were settled when the wife was persuaded by the police to drop the charges (*4,15,16*).

Health workers provide medical assistance to the victims of IPV. However, they do not inquire into the cause of injury. Furthermore, medical officers in Sri Lanka are not trained to address IPV (*4*). In Sri Lanka, an average of over 100 cases of domestic violence is reported in the media every month, although many more go unreported and unrecorded (*4,16*). The laws against domestic violence provide a 12 months protection order against the acts of physical and psychological violence by husbands (*20,21*). However, the poor monitoring after issuing of the protection order leads wives to be battered despite the availability of the law (*19*).

5. IPV prevention activities in Sri Lanka

5.1. *Legal interventions*

In 1995, the Sri Lankan government amended the penal code to recognize physical abuse over spouse and marital rape. However, the marital rape was recognized as an offence only when the spouse is judicially separated or when the wife is under 16 years old (*4*). In 2005, a specific legislation against domestic violence was introduced in Sri Lanka. This new legislation provides protection orders against acts of physical violence, and severe psychological violence such as extortion and intimidation by a spouse. It prevents an aggressor from inflicting harm to victims within the home environment, place of employment or at shelters. However, the law on marital rape did not change (*20,21*).

In 1996, in 36 main police stations in Sri Lanka, women's desks were established to receive the complaints of domestic violence and IPV. These desks are headed by female police officers to address the complaints of IPV in gender sensitive manners (*3,4*).

5.2. *Shelters and supportive servicers for IPV victims*

The Ministry of Child Development and Women's Empowerment has established a shelter for the victims of domestic violence in southern Sri Lanka, and plans to establish 14 more centers throughout the country. These shelters temporarily accommodate the victims of IPV till they find a safe place to live, away from the abusive husbands (*19*).

In Sri Lanka, 50 NGOs working on gender issues have got together and formed a forum (The Sri Lanka Women's NGO forum) to function as a lobbying and advocacy body on women's issues and rights in Sri Lanka. Their main focus is to popularize the Beijing platform for action to prevent violence against women

including IPV (*21-24*).

Of these 50 NGOs, two NGOs (Women in Need and Center for Women's Research) are actively involved in IPV prevention activities in Sri Lanka (*21,23*). They have wide networks with regional centers, and support centers across the country. These NGOs provide legal counseling and assistance to the victims of IPV, and maintain 24-hour help lines to assist those victims. Other than conducting community awareness programs on IPV, they have conducted awareness programs for the police officers as well, with the support of the Department of Police in Sri Lanka.

6. Discussion

This study suggested that the prevalence of IPV is high in Sri Lanka. However, most prevalence studies were carried out in health care settings, and they possibly missed the IPV victims who did not come to these health care institutions. Therefore, the actual prevalence of IPV in the country might be higher than that has been reported. So far, only one community based survey has been conducted to assess the prevalence of IPV in Sri Lanka (*7*). That study also has examined only the physical IPV and has not included the psychological or sexual IPV. Therefore, the prevalence of psychological and sexual IPV needs to be evaluated by community-based research in Sri Lanka. Further, a large scale community based study should assess the national prevalence, and the prevalence of IPV among the different ethnic groups in Sri Lanka.

Health care providers and police officers bear a major responsibility in combating IPV in any country (*4*). However, a considerable proportion of medical students and police officers in Sri Lanka believe that IPV is a personal matter in which outsiders should not intervene (*4,10*). This situation can lead the victims of IPV to experience institutional abuse (*19*) and make them lose confidence in the legal and medical systems for their safety.

The recent initiatives such as IPV education programs for police officers and establishing women desks at police stations might improve this situation. However, those initiatives should be evaluated for their effectiveness. Similar IPV education programs should be introduced for the medical professionals and medical students in Sri Lanka. The attitudes of medical students should be improved to acknowledge the cases of IPV in gender sensitive manners, because once graduated from the medical schools they will have to actively manage the cases of IPV.

The amendment of laws to identify physical and psychological IPV is a positive development to prevent IPV in Sri Lanka. Because sexual IPV carry similar adverse effects to physical or psychological IPV (*1,2*), the laws against IPV should be further amended to provide specific provisions against sexual IPV as well. Once protection orders are issued for the cases of IPV, police officers should monitor the abusers and prevent them from further abusing the victims.

The activities by the NGOs to improve the community awareness of IPV will be important to prevent IPV in Sri Lanka. To maintain the sustainability of these initiatives, the government of Sri Lanka also needs to support these NGO activities. Such an effort might help to change the patriarchal attitudes of the Sri Lankan community towards IPV.

In most literature in Sri Lanka, they discuss IPV under the broad topics of violence against women or domestic violence. This tends to underestimate the importance of IPV. Hence, literature should discuss IPV as a separate and specific issue highlighting its importance.

In conclusion, our literature review suggests a high prevalence of IPV in Sri Lanka. Yet the actual prevalence might be even higher than reported. Moreover, a common belief in Sri Lanka is that IPV is a personal matter which outsiders should not intervene. To improve this situation of IPV in Sri Lanka, we recommend well-designed community-based research on IPV in Sri Lanka. The attitudes toward IPV should be improved among the police officers, medical students and the community in Sri Lanka. The laws against IPV should be amended to include provisions on sexual IPV.

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Original Article

One-year follow-up study of post-traumatic stress disorder among adolescents following the Wen-Chuan earthquake in China

Zhiyue Liu[1], Yanfang Yang[1], Yunli Ye[2], Ziqian Zeng[1], Yingjun Xiang[1], Ping Yuan[1,*]

[1] *Department of Epidemiology, West China School of Public Health, Sichuan University, Chengdu, China;*
[2] *Luzhou Medical School, Luzhou, Sichuan, China.*

Summary **Post-traumatic stress disorder (PTSD) is the most common psychological disorder among victims of natural disasters. PTSD prevalence and risk factors among adolescents remain unidentified among victims of the Wen-Chuan earthquake. This study screened survivors to determine the prevalence of PTSD and examined risk factors for PTSD among adolescents at three Wen-Chuan secondary schools. PTSD screening was done using the PTSD Checklist-Civilian version (PCL-C). A generalized estimating equation approach was used to control for repeated measurements in the same individuals and to predict risk factors for PTSD. The study included 1,474 students in grades 7, 8, 10 and 11 from three Wen-Chuan secondary schools at 4, 6, 9, and 12 months after the earthquake. The average age of students was 15.0 (13.0, 16.0) both at the first and the second time point, and 16.0 (14.0, 17.0) at the third and the fourth time point. The screened prevalence of PTSD was 11.2%, 8.8%, 6.8% and 5.7% at 4, 6, 9, and 12 months after the earthquake, respectively. Risk factors for PTSD were: time duration, school location (the proximity of epicenter), grade, nationality, parent injury, and severe property damage. In conclusion, PTSD risk factors are in accordance with previous studies; however, the role of nationality and time duration in post-traumatic stress disorder merits further research.**

Keywords: Post-traumatic stress disorder, earthquake, adolescents, risk factor, generalized estimating equations

1. Introduction

On May 12, 2008, an earthquake measuring 8.0 on the Richter Scale struck Wen-Chuan County, Sichuan, China, leading to 69,226 deaths and 374,643 injured, with 17,923 listed as missing up to September 11, 2008 (*1*). The earthquake not only caused tremendous damage to people's physical health, but also had deleterious consequences for psychological health. The effects on mental health, which often persist long after the disaster, have been shown to vary according to individuals' exposure to, and experiences emanating from, the event. Studies have also indicated that these effects can depend upon various socio-demographic characteristics such as age and gender (*2*). Previous epidemiological studies have explored the psychological well-being of seismic victims (*3*,*4*). It has been shown that after a natural disaster such as an earthquake survivors are susceptible to post-traumatic stress disorder (PTSD) (*5*,*6*), the most common psychiatric condition seen among earthquake survivors (*7*-*9*). Previous investigations have examined post-traumatic stress disorder among survivors after the Wen-Chuan earthquake. Few studies, however, have included large representative samples of secondary school students.

This study examined the prevalence of PTSD among adolescents as well as socio-demographic and post-earthquake variables associated with PTSD, especially the time factor in the trajectory of PTSD.

2. Materials and Methods

2.1. *Subjects*

The study used cluster sampling. We selected all the students from grades 7, 8, 10, and 11 in three secondary

**Address correspondence to:*
Dr. Ping Yuan, Department of Epidemiology, West China School of Public Health, Sichuan University, Chengdu 610041, China.
e-mail: yuanp1117@hotmail.com

schools (Wei-Zhou, Mian-Chi, Qi-Pan-Gou) originally located in Wen-Chuan County, who resumed their classes in the suburbs of Cheng-Du City after the disastrous earthquake. Grades 9 and 12 were excluded due to the pressures of entering middle school and college. We conducted the baseline survey at about 4 months after the earthquake and follow-up surveys at 6, 9, and 12 months after the earthquake. Students who transferred from other schools after the Wen-Chuan earthquake were excluded. These investigations, with the class as the unit, were conducted by eight well-trained and experienced investigators with training in epidemiology who received unified training about how to explain items in self-administered questionnaires and to keep students from talking to each other when filling out the questionnaire. The investigation process took about thirty minutes per class.

2.2. *Measures*

In the baseline study, background data such as gender, nationality, place of residence before earthquake, personal and parent injury, hospitalization, and severe property damage in earthquake were collected; the PTSD instrument used in the study was the PTSD Checklist-Civilian version (PCL-C). The same PTSD instrument was used in follow-up studies.

The PTSD Checklist-Civilian version (PCL-C) (*10*) is a 17-item self-reported rating-scale instrument ranging from 1 (not at all) to 5 (extremely) that measures symptoms of PTSD in the past month. The PCL parallels diagnostic Criteria B, C, and D for PTSD, as delineated in the DSM-IV (*11*). There are two alternative ways of PCL scoring: one is based on endorsement (a symptom rating of at least 3) of one or more "reexperiencing" symptoms (items 1-5), three "avoidance" symptoms (items 6-12), and two "arousal" symptoms (items 13-17); the other, the most commonly used method of scoring, involves summing the responses from the 17 items yielding a score ranging from 17 to 85 and selecting a cutoff within this range. A cutoff of either 48 or 50 resulted in an estimated prevalence of PTSD that matches the true prevalence most closely (*12*). A score of 50 is considered the optimally efficient PTSD cutoff score which provides good diagnostic sensitivity (0.82) and specificity (0.83), with a kappa coefficient of 0.64 (*13,14*). Several psychometric studies have supported the reliability and validity of the PCL with a variety of samples (*10,15,16*), and the PCL has been previously used with adolescents (*17-19*), which is an indication of its support in this study group.

2.3. *Statistical analysis*

The data were analyzed on a personal computer with SAS software (version 9.1, SAS Institute, Inc., Cary, NC). Missing values were compensated by using the average score of each symptom if only 1 or 2 items were missing; data were considered invalid if 3 or more items were missing. Variables were tested for normal distribution. All values were reported as medians for partially distributed variables. All p values were two-sided; a p value < 0.05 was considered statistically significant.

In the first step, we tested whether PTSD or other demographic variables were predictive of dropout, to find out whether there were any sample differences between the baseline and follow-up investigations which would have impacts on data analysis. Using logistic regression, we ran separate models for each predictor and adjusted for drop-out status (drop-out or follow-up) on sex, grade, nationality, residence, personal injury, personal hospitalization, parent injury, severe property damage, and PTSD (a total PCL-C score of > 50 was encoded as 1, < 50 as 0).

The impact of several variables on PTSD was analyzed using regression modeling by a generalized estimating equation to control for repeated measurements in the same individuals. Analyses were conducted in the GENMOD procedure, with logit link and an autoregressive correlation matrix. Ten variables were entered into GEE: time point (1, 2, 3, 4), school location (Wei-Zhou, Mian-Chi, Qi-Pan-Gou), grade (7th, 8th, 10th, 11th), gender (male, female), nationality (Han, Minorities), residence (urban, rural), personal injury (yes/no), personal hospitalization (yes/no), parent injury (yes/no), and severe property damage (yes/no).

3. Results

3.1. *Study population*

Overall, 1,966 students were eligible to participate; 1,966, 1,928 (98.1%), 1,823 (92.7%) and 1,842 (93.7%) questionnaires were received respectively at 4, 6, 9, and 12 months after the earthquake, of which 1,960 (99.7%), 1,925 (99.8%), 1,511 (82.9%) and 1,840 (99.9%) were valid; 1,490 (75.9%) students completed all four questionnaires and 1,474 were valid (some students did not complete all four questionnaires due to sick leave or home visits at the time of the investigation). Tests of normal distribution showed that age was partially normally distributed; thus the median (QL, QU) was used to describe the distribution. The students' average age was 15.0 (13.0, 16.0) both at the first and the second time point, and 16.0 (14.0, 17.0) at the third and the fourth time point. Demographic characteristics of subjects are summarized in Table 1.

3.2. *Dropout analysis*

To determine the extent to which baseline measures predicted subsequent attrition, a dichotomous indicator

Table 1. Socio-demographic characteristics of subjects

Characteristic	No.	Proportion (%)
Gender		
Male	663	45.0
Female	811	55.0
School location		
Wei-Zhou	907	61.5
Mian-Chi	249	16.9
Qi-Pan-Gou	318	21.6
Grade		
7th	391	26.5
8th	262	17.8
10th	390	26.5
11th	431	29.2
Nationality		
Han	175	11.9
Minorities	1,299	88.1
Residence		
Urban	639	43.4
Rural	835	56.6
Personal injury		
Yes	69	4.7
No	1,405	95.3
Personal hospitalization		
Yes	14	0.9
No	1,460	99.1
Parent injury		
Yes	153	10.4
No	1,321	89.6
Severe property damage*		
Yes	1,254	85.1
No	220	14.9
Total	1,474	100

*Severe property damage refers to: a, place of residence rendered uninhabitable by damage or demolition; or b, major loss of money or destruction of expensive appliances in the earthquake.

of dropout was created, and logistic regression was used. None of the predictors approached statistical significance ($p > 0.05$).

3.3. *PTSD prevalence and risk factors*

The proportion of participants who met screening criteria (50-point cutoff) for PTSD was calculated: it was 11.2% ($n = 165$) of students in the baseline study, 8.8% ($n = 129$) at 6-month follow-up, 6.8% ($n = 100$) at 9-month follow-up, and 5.7% ($n = 84$) at 12-month follow-up.

By means of a generalized estimating equation, the positive rate of PTSD at time point 1 (OR = 2.13, 95% CI = 1.66-2.73) and time point 2 (OR = 1.6, 95% CI = 1.27-2.02) were found to be significantly different compared with time point 4, as shown in Table 2; no statistical significance was found between rates at time points 3 and 4 ($p > 0.05$). Students in Mian-Chi Secondary School had higher odds of PTSD (OR = 3.16, 95% CI = 2.01-4.97). Students in the 8th, 10th, and 11th grade all had higher odds of PTSD than students in the 7th grade; in the 10th grade, values were the highest (OR = 3.08, 95% CI = 1.48-6.43). Students who belonged to minorities (OR = 1.77, 95% CI = 1.06-2.94), whose parent was injured (OR = 1.67, 95% CI = 1.14-2.45), or whose parent suffered severe property damage (OR = 2.12, 95% CI = 1.21-3.72) were more likely to be found through screening to have PTSD. Associations among variables such as gender, residence, personal injury, and personal hospitalization showed no statistical significance ($p > 0.05$).

4. Discussion

4.1. *PTSD prevalence and time as a risk factor*

Current estimates of the prevalence of PTSD in trauma populations are highly diverse: some illustrative figures range from 5% to 8% among victims of some natural disasters (*20*). The prevalence of PTSD among Chi-Chi earthquake survivors decreased from 8.3% at 6 months to 4.2% at 3 years after the earthquake (*21*). In this study, the prevalence of PTSD was 11.2%, 8.8%, 6.8%, and 5.7% at 4, 6, 9, and 12 months after the earthquake, respectively. However, the PTSD prevalence was lower than in other studies; this is presumably due mainly to diverging focuses on different categories of victims or the use of different instruments to measure PTSD (*22*,*23*). By choosing the cutoff of 50, the estimated prevalence of PTSD in our study may be closest to the actual prevalence, according to Artin Terhakopian's research (*12*).

Adolescents had relatively high chances of suffering PTSD proximately, that is, four months after the earthquake, with an OR of 2.13; the OR of PTSD six months after the earthquake was 1.6, and 1.2 nine months after the earthquake. Although no statistical significance was found at the 9th month compared to the other time points, we found that the prevalence of PTSD in secondary school students declined in this one-year follow-up study, and this decrease slowed as time went by. Previous studies indicate that most cases of spontaneous recovery from PTSD occur within the first year following exposure to the traumatic event, whereas an unremitting course is expected whenever the disorder is present for more than 5 years (*24*,*25*). Other studies also show a decrease in PTSD cases with time (*23*,*26*). Some studies have shown that the psychological consequences of earthquakes can be long-term (*27*,*28*), and the notion that PTSD symptoms decrease with time has been challenged by long-term studies (*29*,*30*). Therefore, although the prevalence of PTSD tended to decline in the first year following the Wen-Chuan earthquake, it is nonetheless possible that in the long run, PTSD will become chronic in some adolescents, which may significantly affect

Table 2. PTSD risk factors predicted by means of a generalized estimating equation

Background factors	Estimate	SE	*p*	OR	95% CI
Time points					
Time point 1	0.755	0.127	< 0.0001	2.13	1.66-2.73
Time point 2	0.471	0.117	< 0.0001	1.60	1.27-2.02
Time point 3	0.185	0.113	0.102	1.20	0.96-1.50
Time point 4	0.000			1.00	
School location					
Wei-Zhou	– 0.208	0.397	0.601	0.81	0.37-1.77
Mian-Chi	1.151	0.230	< 0.0001	3.16	2.01-4.97
Qi-Pan-Gou	0.000			1.00	
Grade					
8th grade	0.521	0.210	0.013	1.68	1.12-2.54
10th grade	1.125	0.375	0.0027	3.08	1.48-6.43
11th grade	1.057	0.382	0.0056	2.88	1.36-6.08
7th grade	0.000			1.00	
Gender					
Female	0.157	0.137	0.254	1.17	0.89-1.53
Male	0.000			1.00	
Nationality					
Minorities	0.569	0.259	0.028	1.77	1.06-2.94
Han	0.000			1.00	
Residence					
Rural	0.0003	0.170	0.998	1.00	0.72-1.40
Urban	0.000			1.00	
Personal injury					
Yes	0.475	0.298	0.111	1.61	0.90-2.88
No	0.000			1.00	
Personal hospitalization					
Yes	0.442	0.472	0.349	1.56	0.62-3.92
No	0.000			1.00	
Parent injury					
Yes	0.511	0.196	0.009	1.67	1.14-2.45
No	0.000			1.00	
Severe property damage					
Yes	0.752	0.287	0.009	2.12	1.21-3.72
No	0.000			1.00	

their psychological development. For example, in one study, Karakaya *et al*. have found very severe or severe degrees of post-traumatic stress symptoms in 22.2% of adolescents even three and a half years after the 1999 earthquake in Turkey (*30*).

4.2. *Different school location as a risk factor*

The present study showed that students in Mian-Chi Secondary School had a higher risk of PTSD. The reason is that Mian-Chi Secondary School is the nearest to the epicenter, in Ying-Xiu Township (the distance from epicenter to school in descending order is Mian-Chi, Qi-Pan-Gou, and Wei-Zhou). It was reported that proximity to the epicenter was the most powerful predictor of PTSD in the 1989 San Francisco earthquake (*31*), and that children living closest to the epicenter suffered from severer PTSD symptoms than those living further from the epicenter in the 1988 Armenian earthquake (*32*). In addition, Mian-Chi Secondary School was hit by a boulder rolling down from the hills during the earthquake, killing 12 students and causing more than 20 injuries. In our investigation, the principal told us that all the students at Mian-Chi Secondary School had witnessed the tragedy. It has been proved that PTSD can occur in persons who have witnessed a violent injury or the unnatural death of another person (*33*).

4.3. *Grade as a risk factor*

Studies of disaster victims have highlighted the

influence of previous experiences and mental states on vulnerability to PTSD. For instance, it has been reported that the severity of PTSD symptoms in child survivors of a shipwreck was associated with mental problems existing before the disaster (*34*). Stressful life events occurring subsequent to the disaster were also related to the severity of PTSD (*35*). In our study, senior middle school students (*i.e.,* students in the 10th and 11th grades) had a higher risk of PTSD than junior middle school students (*i.e.,* students in the 7th and 8th grades). This suggests that senior middle school students might feel greater pressure due to their heavier study load than junior middle school students. The fact that students in the 10th grade had a higher risk than 11th grade students could be explained by presuming that before the earthquake occurred, students who were in the last stage of 9th grade (*i.e.,* the 10th grade in our present study) may have undergone great pressure while preparing to take their entrance exams for senior middle school; after the earthquake, the previous 9th grade students entered the 10th grade (the first year in senior middle school); thus students in the 10th grade may have faced greater difficulties in dealing with their new study environment, which in turn, could result in higher PTSD prevalence.

4.4. *Other factors related to PTSD*

As suggested in some studies, serious earthquake damage to the home and property is associated with psychological problems (*4*,*36*). Our study was consistent with this finding. Although a parent's injury has not been studied as a factor in previous research, this factor turned out to be one of the risk factors identified in our study. Female gender has been associated with PTSD in adolescents in some studies (*34*,*37*) but not in others (*38-40*). No statistically significant difference between boys and girls was found in PTSD prevalence in this study. The literature suggests that personal physical injury is a risk factor for the development of PTSD (*41*). However, in this study, personal injury was not significantly associated with PTSD. Presumably, the small number of injured students is largely responsible for the lack of significance. It may be assumed that, with a relatively large number of injured subjects, this factor would have become significant. Nationality was another risk factor of PTSD in our study: the Minority students had a greater risk of PTSD than the Han students. It may be postulated that the Han people could get more social support from the outside world, as most of the Han families had emigrated to Wen-Chuan for the sake of work and therefore could utilize a wider sphere of social resources after the earthquake. A search of the literature turned up no studies on differences in psychological morbidity between Han nationality and Minorities; further research is called for to investigate this phenomenon.

4.5. *Limitations and strengths*

Our study is subject to certain limitations. Firstly, the subjects of our study moved to the suburbs of Cheng-Du after the earthquake due to damage to their schools in Wen-Chuan. Hence, it is possible that the new school environment and great distance from family could have certain negative impacts on the subjects, which might also cause or aggravate PTSD. Secondly, some of the earthquake experiences were relatively rare. For example, only 0.9 percent of students were hospitalized and 4.7 percent of students were injured; this limited the statistical power to predict some of the risk factors of PTSD.

Despite those limitations, this study, to the best of our knowledge, is the first large-scale study with a sample of 1,474 students to estimate the prevalence of PTSD among adolescents at about 4, 6, 9, and 12 months after the Wen-Chuan earthquake. Another highlight of our study is the specific analysis of data by a generalized estimating equation to control for repeated measurements in the same individuals and to predict risk factors effectively.

5. Conclusion

In conclusion, the prevalence of PTSD in adolescents was 11.2%, 8.8%, 6.8%, and 5.7% at 4, 6, 9 and 12 months, respectively, after the earthquake. The risk factors of PTSD were time duration, school location (proximity to the epicenter), school grade, nationality, parent injury, and severe property damage during the earthquake. How nationality affected the development of PTSD deserves further study. In addition, long-term longitudinal studies are needed to clarify the role of time in the trajectory of PTSD.

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Original Article

DNA content can improve the detection and prognosis of carcinoma of the cervix

Chhavi[1,*], Mona Saxena[2], Mahendra P. S. Negi[3], Sharad Singh[1], Pankaj K. Singh[1], Urmila Singh[4], M. L. B. Bhatt[1]

[1] *Department of Radiotherapy, C.S.M. Medical University, Lucknow, India;*
[2] *Department of Biochemistry, Career Post Graduate Institute of Dental Sciences and Hospital, Lucknow, India;*
[3] *Biometry and Statistics Division, Central Drug Research Institute, Lucknow, India;*
[4] *Queen Mary's Hospital, Dept. of Obstetrics and Gynecology, C.S.M. Medical University, Lucknow, India.*

Summary **In all malignant diseases, several clinical and histopathological tests are established as standard methods for diagnosis. Alternative diagnostic quantitative methods are still lacking or conflicting in cancer of the cervix. Although DNA analysis was one of the earliest applications in flow cytometry and the DNA content of fresh/frozen tissue have shown good prognostic results in many diseases including cervical cancer, prognostic ability cannot be easily clarified quantitatively by biochemical values. Thus, our objective was to determine whether the addition of a DNA content study using flow cytometry improves the detection of cervix cancer. The quantification of DNA content was done by high resolution DNA flow cytometry in fresh/frozen tissues of healthy cervix (control, $n = 38$) and from cancer of the cervix ($n = 62$). Results show that the mean fraction of Total S phase, Total Aneuploid and G2-M (Diploid) are significantly higher ($p < 0.01$); while G0-G1 (Diploid) and G0-G1 (Aneuploid) are significantly lower ($p < 0.01$) in cancer patients as compared to control. Among cell cycle parameters, G0-G1 (Diploid) shows a classifying ability of 97% and at a criterion/threshold value of ≤ 79.88, it discriminates cases with the highest sensitivity of 96.77 (88.8-99.5; 95% CI) and specificity 100.0 (90.7-100.0; 95% CI) and with Total S phase or Aneuploid, it discriminates cases with 100% sensitivity. G0-G1 (Diploid) also showed a direct and significant correlation ($r = 0.66$; $p < 0.01$) with patient survival indicating prognostic significance. Cell cycle parameters will be very helpful in false positive results or where the clinician is unable to diagnose the stage of cervix cancer clinically or histopathologically.**

Keywords: DNA content, G0-G1 (Diploid), G0-G1 (Aneuploid), Total S phase, flow cytometry

1. Introduction

Despite the introduction of a number of organized and ad-hoc screening test programs, cervical cancer continues to remain a major source of mortality and morbidity for women in developing countries and is still the second most common cancer among women worldwide, especially in middle and low income countries. In India, about 130,000 new cases of cervix cancer occur every year and constitute one-fifth of the total global burden. The age-standardized incidence rate is 30.7 per 100,000 and age-standardized mortality rate is 17.4 per 100,000 in India, which is the highest in South Central Asia (*1*). In all malignant diseases, several clinical and histopathological tests are established as standard methods for diagnosis. Alternative quantitative methods of diagnosis are available in many other cancers but they are still lacking or conflicting in cervix cancer. Cytomorphologically (Pap test) abnormal epithelial cells of cervical smears are generally used for screening. Unfortunately, a substantial number of premalignant and malignant lesions of the uterine cervix have a history of normal cervical smears. The false negative rate of diagnosis varies considerably

**Address correspondence to:*
Dr. Chhavi, c/o Prof. Madanlal B. Bhatt, Department of Radiotherapy, C.S.M. Medical University, Lucknow, India.
e-mail: chhavi121@gmail.com (Chhavi), drmlbhatt@yahoo.com (Bhatt M)

between 15 and 50%, while the number of false positive is about 10% (*2*). Several ACCP (The Alliance for Cervical Cancer Prevention) studies have also found Pap test sensitivity in the range of 50% at best (*3*). Thus, limitations of these cytological tests as moderate to low sensitivity and false-positive results leading to unnecessary patient anxiety and costs, limits the usefulness of Pap examination as a sensitive tool for early diagnosis (*2*). Beside early diagnosis, staging is another important feature in the management of cancer, as it guides the mode of treatment and adjuvant therapy thereafter. Staging itself is a very challenging task and there is a marked inter-examiner variability for the same patient as staged by different clinicians. However, the present day treatment decisions are being made solely on the FIGO stage of the patient. Despite the sophisticated diagnostic techniques, it is not always possible to predict the individual proliferative potential and tendency of recurrence among tumors within the same histological subtypes. Even patients with tumors confined only to the cervix *i.e.*, stage IA or B develop vault recurrence in as many as 20% cases after the various treatment modalities (*4*). New biological parameters are, therefore, needed for more accurate diagnosis requiring more aggressive and individualized treatment schedules.

Although various DNA flow cytometric studies have been performed on cervix cancer, the role of DNA ploidy and the S-phase fraction (SPF) in predicting biological tumor behavior finally remains unresolved. Although DNA analysis was one of the earliest applications of flow cytometry and the DNA ploidy content have shown prognostic ability in many cancer diseases such as cervical cancer (*5*), head and neck (*6*), ovarian (*7*), colon (*8*), and endometrial (*9*), but the prognostic ability cannot be easily clarified quantitatively in cervical cancer. The threshold values are provided but only for S phase and not for other cell cycle parameters. One main drawback of these studies was that the researchers were trying to identify diagnostic and prognostic indicators within an identical subgroup of cancer patients. Keeping the above discrepancy and controversy in mind, the present study was designed to determine the diagnostic accuracy of the disease by correlating pathological and clinical parameters with cell cycle parameters and evaluating them by correlating these with patient survival time. Thus, our objective was to determine whether the addition of a DNA content study using flow cytometry improves the detection of cervical cancer.

2. Material and Methods

2.1. *Patient selection and sample collection*

The present study was done in clinically diagnosed and histopathologically confirmed 62 cases of cervical carcinoma and 38 control cases (sample size, $n = 100$). The cases were selected from the patients registered in the Department of Radiotherapy and Gynecology, Chhatrapati Shahuji Maharaj Medical University, Lucknow during the years 2004-2006. Criteria for choosing patients with carcinoma of the cervix for inclusion in the study were previously untreated patients without any concurrent malignancy or chronic illness and with normal renal and liver function tests. The control population consisted of individuals undergoing hysterectomy for benign conditions. The age of the subjects varied from 22 to 76 years (mean age 47.12 ± 11.12 years). All procedures were performed in accordance with our hospital's ethical guidelines, and approval for the study was granted by the university hospital's ethics committee. The voluntary written consent was taken from all the subjects for participation in the study. A detailed history and clinical examination was undertaken for all subjects in the study. The samples for the suspected cervix cancer (malignant) cases were collected when the biopsy was taken to confirm diagnosis. The biopsies were guided by colposcopy and taken at the squamous-columnar junction. The samples for the control cases were collected when the hysterectomy was performed and was being sent for pathological examination. All the cervical biopsy samples were collected in phosphate buffered saline and cases were finally classified in different groups according to the FIGO staging (*10*).

2.2. *Histopathological evaluation*

Histopathological examinations were done on hematoxylin and eosin stained sections. All selected cases were positive for squamous cell carcinoma of the cervix except controls. Histopathological examination of cervix for controls exhibited a benign normal appearance. The squamous cell carcinoma (SCC) cases were classified on the basis of a modified Broder's classification (*11*) and graded according to the degree of differentiation as well, moderately and poorly differentiated. According to the findings of clinical and histopathological examinations of patients they were scored with dummy numbers from 0 to 6 along with a control according to the disease progression and/or severity of carcinoma. Out of 8 clinical sub-stages of cancer, this study involved only patients of 6 sub-stages because during the study period no cases of initial (IA) and last sub-stage (IVB) patients were found. 8 patients were found in IB group, 2 in IIA, 19 in IIB, 1 patient in IIIA group, 30 patients in IIIB group, and 2 patients in IVA group. The groups were graded as 1, 2, 3, 4, 5 and 6, respectively.

2.3. *Methodology for preparation of single cells*

Samples for flow cytometric evaluation were prepared

with some modifications of the method originally described by Vindelov *et al*. (*12*) and MEDLINE search (*13*) which was finally standardized in our laboratory. To standardize the preparation of single cell suspension, different concentrations of trypsin were tried**.** The tissue was minced with curved scissors, and transferred in five different tubes. Each tube was then treated with 1 mL of trypsin (3× purified, SRL) in 1 N HCl solution at concentrations of 0.05%, 0.10%, 0.25%, 1.0% and 2.5%, respectively. The tubes were incubated at 37°C and mixed intermittently for 10-15 min. Cells were then gently dispersed by triturating (triturating: cell dispersion through mild pumping action). The suspension was then filtered through fine mesh. Cells were then allowed to settle and excess liquid containing enzymes was decanted after centrifugation at 500 × g. The pellet was then washed 2-3 times. Finally, the single cell suspension was obtained by re-suspending cells in ice-cold PBS buffer (pH 7.4). After the acquisition of all these suspensions, the suspension with 2.5% concentration of trypsin was found to be the best for control tissues and 0.05% for cancerous tissues. Suspensions of all tissue samples were prepared as above using 2.5% and 0.05% concentrations of trypsin. Flow cytometric acquisition and analysis followed.

2.4. *Flow cytometric analysis*

A standard FACS Calibur (BD Biosciences, San Jose, CA, USA) equipped with a 488-nm argon laser and four-color filters was used to analyze the cellular DNA content using a standard protocol with the appropriate filter combinations for excitation and measurement of the fluorescence of propidium iodide with wavelength settings of 457 nm (FL1), 568 nm (FL2) and 645 nm (FL3) band-pass fluorescence filters and a 781 nm red diode laser with a 684 nm band-pass filter (FL4). A total of 10,000 events were analyzed by flow cytometry using an excitation wavelength set at 488 nm and its fluorescence was detected from 550 up to 670 nm. Cells present in single cell suspension of all the tissue samples were fixed with chilled ethanol for 30 min at 4°C. After adding propidium iodide (50 µg/mL), this mixture was stored in the dark for 1 h at room temperature before additional analysis. At least 10,000 events were counted in each sample. The cell cycle profiling of all samples was done in the same environment using the same instrument and by the same observer. Human lymphocytes were used as an external standard or for calibration. DNA histogram cell-cycle analysis was performed as described by Rabinovitch (*14*) using ModFit LT for Mac V2.0 software. For each sample, the percentage distribution of cells in the following 12 Cell Cycle Parameters *viz*.: Total S phase, Total Diploid, Total Aneuploid, DNA index (DI), G0-G1 (DIP; Diploid), G2-M (DIP), S (DIP), G2/G1 (DIP), G0-G1 (ANP; Aneuploid), G2-M (ANP), S (ANP) and G2/G1 (ANP) were estimated.

2.5. *Statistical analysis*

The overall (n = 100) average (± SE) coefficient of variation (COV) of diploid was 0.14 (± 0.01) while that of aneuploid was 0.23 (± 0.14). The respective value of these in controls (n = 38) was 0.10 ± 0.005 and 0.04 ± 0.004, respectively, while those in patients (n = 62) were 0.17 ± 0.01 and 0.35 ± 0.22, respectively. Seven parameters *i.e*., Diploid, DI, S (DIP), G2/G1 (DIP), G2-M (ANP), S (ANP) and G2/G1 (ANP) were not found statistically significant and either were not correlated ($r \leq 0.50$) well with clinical and histopathological gradings or found to be similar, and were excluded from further analysis. Thus, five total parameters *i.e*., Total S phase, Aneuploid, G0-G1 (DIP), G2-M (DIP) and G0-G1 (ANP) which were found clinically significant were submitted for statistical analysis. Cell cycle parameters of two groups were compared by independent Student's *t*-tests. Classification of cases were done using binary logistic regression while diagnostic, by receiver operating characteristic (ROC) curve analysis (Figure 2). Association between variables was done by Pearson correlation coefficient (r). Comparison of survival curve, was done by Log rank test. MS EXCEL (MS Office 97-2003), Graph Pad Prism (Version 5) and Med Calc (Version 3) were used for the analysis. A two-tailed ($\alpha = 2$) probability (p) value less than 0.05 ($p < 0.05$) was considered to be statistically significant.

3. Results

The baseline cell cycle parameters of control and patients are summarized in Table 1. Table 1 shows that the average fraction of Total S phase, Aneuploid

Table 1. Summary of DNA content of controls and patients

Parameters	Control (n = 38)				Patients (n = 62)				t-value (DF = 98)
	Min	Max	Mean ± SE	COV	Min	Max	Mean ± SE	COV	
Total S phase	0.00	11.44	5.47 ± 0.52	0.58	2.62	80.06	32.23 ± 2.47	0.60	8.39*
Aneuploid	0.02	27.82	4.91 ± 0.83	1.04	0.00	94.35	37.58 ± 3.23	0.68	7.80*
G0-G1 (DIP)	82.82	99.18	94.01 ± 0.62	0.04	0.00	89.72	30.77 ± 4.05	1.04	12.16*
G2-M (DIP)	0.00	17.18	0.65 ± 0.46	4.29	0.00	95.93	35.17 ± 4.64	1.04	5.81*
G0-G1 (ANP)	0.00	100.00	85.99 ± 5.00	0.36	0.00	100.00	39.48 ± 4.44	0.89	6.74*

* $p < 0.01$.

and G2-M (DIP) in patients were significantly high ($p < 0.01$) while G0-G1 (DIP) and G0-G1 (ANP) were significantly low ($p < 0.01$) as compared to control. Representive histograms of each group are shown in Figure 1. Corrected classifications (%) of cases of two groups are summarized in Table 2. All cell cycle parameters had high diagnostic value as they classified together (discriminated) control and cancer (patient) cases 100% correctly. Among these parameters, G0-G1 (DIP) alone classified cases 97% correctly, and with Total S phase or Aneuploid it discriminated cases 100% correctly. The percentage of correct classification using each parameter was higher in controls than in patients except Total S phase. The diagnostic value of cell cycle parameters or the accuracy of cell proliferation to discriminate controls and patients are summarized in Table 3. Among the cell cycle parameters, G0-G1 (DIP) showed the highest area under the curve (AUC = 0.997; SE = 0.007; 95% CI = 0.957 to 1.000; Z = 73.743; $p < 0.0001$), which indicates its highest diagnostic benefit, and at a criterion (threshold) value of ≤ 79.88 it discriminated the cases of two groups with

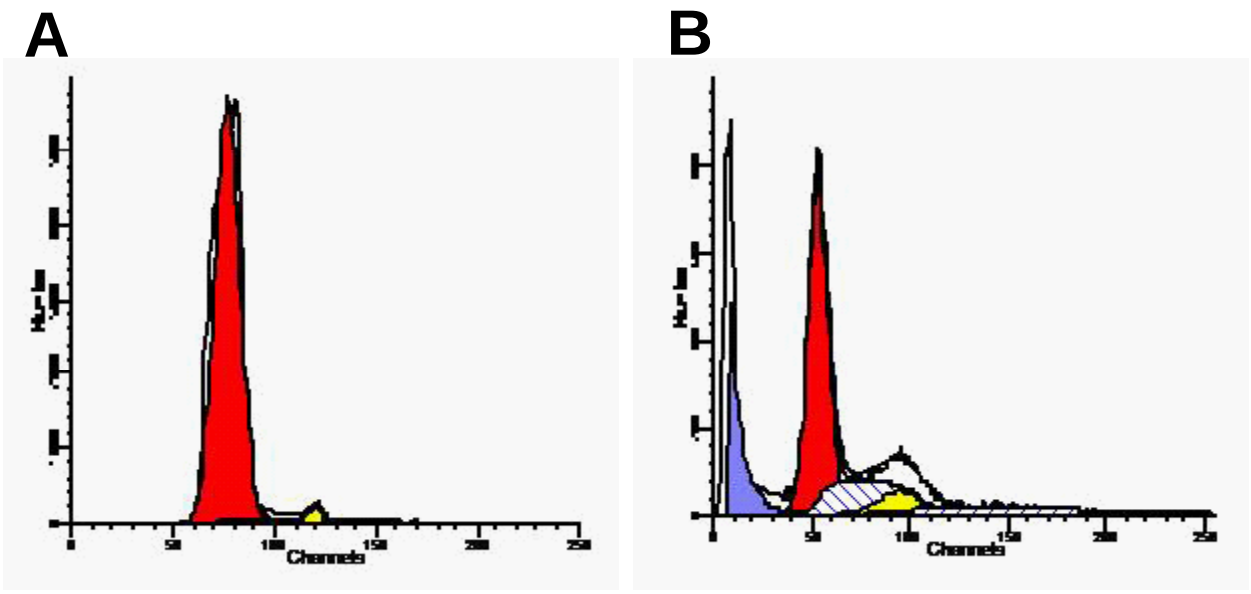

Figure 1. Typical patterns of flow cytometric histogramas of control and malignant cervical tissue in single cell suspension. **A**, Control; **B**, Cancer.

Table 2. Correct classification (%) of all cases, controls and patients by DNA content

Parameters	Control ($n = 38$)		Patients ($n = 62$)		Total ($n = 100$)	
	Number correct	Percent correct	Number correct	Percent correct	Number correct	Percent correct
Total S phase	33	86.8%	56	90.3%	89	89.0%
Aneuploid	34	89.5%	53	85.5%	87	87.0%
G0-G1 (DIP)	37	97.4%	60	96.8%	97	97.0%
G2-M (DIP)	36	94.7%	43	69.4%	79	79.0%
G0-G1 (ANP)	33	86.8%	49	79.0%	82	82.0%
All	38	100.0%	62	100.0%	100	100.0%
G0-G1 (DIP) and Total S phase	38	100.0%	62	100.0%	100	100.0%
G0-G1 (DIP) and Aneuploid	38	100.0%	62	100.0%	100	100.0%

Table 3. Diagnostic of all cases by DNA content ($n = 100$)

Parameters	AUC	Criterion	Sensitivity (95% CI)	Specificity (95% CI)	Z-Statistic
Total S phase	0.949	> 11.44	88.71 (78.1-95.3)	100.00 (90.7-100.0)	21.15*
Aneuploid	0.932	> 11.26	83.87 (72.3-92.0)	94.74 (82.2-99.2)	17.41*
G0-G1 (DIP)	0.997	≤ 79.88	96.77 (88.8-99.5)	100.00 (90.7-100.0)	73.74*
G2-M (DIP)	0.827	> 2.88	69.35 (56.3-80.4)	97.37 (86.1-99.6)	8.10*
G0-G1 (ANP)	0.844	≤ 80.59	82.26 (70.5-90.8)	86.84 (71.9-95.5)	7.91*

* $p < 0.01$.

the highest sensitivity (96.77%) and specificity (100%) as similarly shown from ROC curve analysis (Figure 2). The correlation between gradings of malignancy and P/V examination findings of clinical, biopsy reports and cell cycle parameters of all cases are summarized in Table 4. Table 4 showed that all cell cycle parameters are significant and correlated well with the gradings of clinical and histopathological examinations ($p < 0.01$) and the correlation with both clinical parameters were higher than the histopathological findings. Correlation of two-year survival of the patients (excluding 12 censored cases) with their pre-clinical, histopathological and cell cycle parameters was examined. As shown in Table 5, pre-clinical and histopathological parameters are not significant and correlated ($p > 0.05$) with survival, while the cell cycle parameters especially Aneuploid, G0-G1 (DIP) and G2-M (DIP) are significant and correlated with survival at $p < 0.05$ or $p < 0.01$. A hypothetical two-year overall (including censored cases) survival (%) was investigated in patients with G0-G1 (DIP) ≤ 25 and > 25. The median survival of patients with G0-G1 (DIP) > 25 was found to be 24 months while that with ≤ 25 was 14 months (Figure 3). The median survival of patients with G0-G1 (DIP) > 25 was 1.7 times more and significantly higher than that with ≤ 25 ($p < 0.01$).

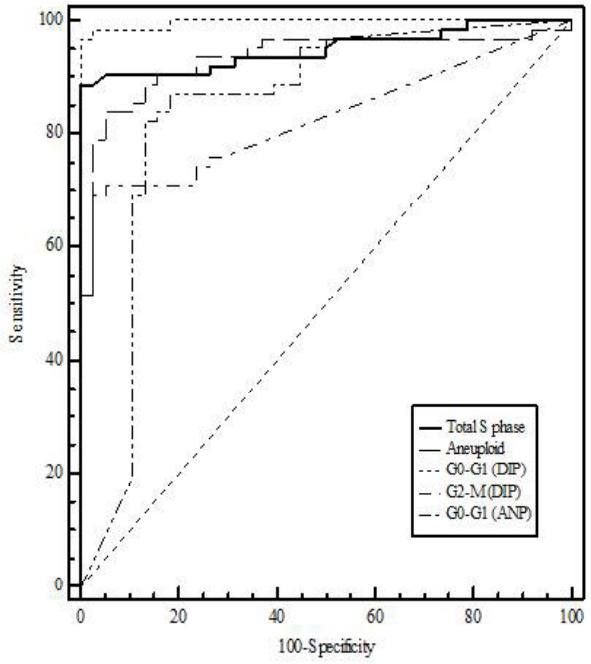

Figure 2. Comparative diagnostic of cell cycle parameters from controls and patients (n = 100) by ROC curve analysis. Different lines represent the sensitivity and specificity of cell cycle parameters. Here G0-G1 (DIP) and Total S phase shows the highest 100% specificity and sensitivity of 96.77% and 88.77%, respectively.

4. Discussion

Clinical staging has been the most important single parameter influencing the choice of treatment as well as outcome. However, clinical staging has some shortcomings and allows significant variations in tumor volume within stage. The challenge in the last few years has been to find a measurable prognosticator which can identify biologically more aggressive cervical cancer. In contrast to many conventional prognostic factors such as lymphnode metastasis, size of primary tumor, myometrial invasion, lymphovasucular involvement, tumor volume, cell type, grade of differentiation, vessel invasion, microvessel density (*15*,*16*), nuclear DNA content can be measured objectively and reproducibly. This is an important criterion in the prognostic factor evaluation outlined by McGuire in 1991 (*17*). In the present study, ROC curve analysis of all the above cell cycle parameters further gives the threshold value for G0/G1 (Diploid), Total S phase, Total Aneuploid involving the quantitative value which can more precisely differentiate and predict the disease. However, G0/G1 (Dipoid) values in the present study was the most sensitive parameter among all other cell cycle parameters such as, Total S phase, S (Diploid), S (Aneuploid), Total Diploid, Total Aneuploid, and DNA index as estimated in all other studies. However, as the restriction point that marks the first transition from G1 to S phase, the other being from metaphase to anaphase (the spindle checkpoint) and between anaphase and telophase when mitotic cyclins are degraded, the "G0-G1; diploid" and "G0-G1; aneuploid" may show no significant difference between control and patients. This can occur, because "cancer" can override restriction points (R-Point) on cell cycles and consequently can avoid apoptosis or

Table 4. Correlation between clinical and histopatological gradings and DNA content of all cases (n = 100)

Parameters	Sub-stage	Exam P/V	Biopsy report	Total S phase	Aneu ploid	G0-G1 (DIP)	G2-M (DIP)	G0-G1 (ANP)
Sub-stage	1.00							
Exam P/V	0.96**	1.00						
Biopsy report	0.50**	0.65**	1.00					
Total S phase	0.58**	0.63**	0.49**	1.00				
Aneuploid	0.62**	0.64**	0.42**	0.47**	1.00			
G0-G1 (DIP)	– 0.76**	– 0.79**	– 0.59**	– 0.64**	– 0.79**	1.00		
G2-M (DIP)	0.52**	0.53**	0.42**	0.22*	0.38**	– 0.66**	1.00	
G0-G1 (ANP)	– 0.43**	– 0.50**	– 0.50**	– 0.42**	– 0.05ns	0.30**	– 0.48**	1.00

ns, not significant; *, $p < 0.05$; **, $p < 0.01$.

Table 5. Corrletion of survival time (month) with clinical and histopatological gradings and DNA content of patients (n = 62)

Parameters	Correlation (DF = 60)
Sub-stage	-0.14^{ns}
Exam P/V	-0.10^{ns}
Biopsy report	0.08^{ns}
Total S phase	-0.01^{ns}
Aneuploid	-0.45^{**}
G0-G1 (DIP)	0.66^{**}
G2-M (DIP)	-0.29^{*}
G0-G1 (ANP)	-0.33^{**}

^{ns}, not significant; *, $p < 0.05$; **, $p < 0.01$.

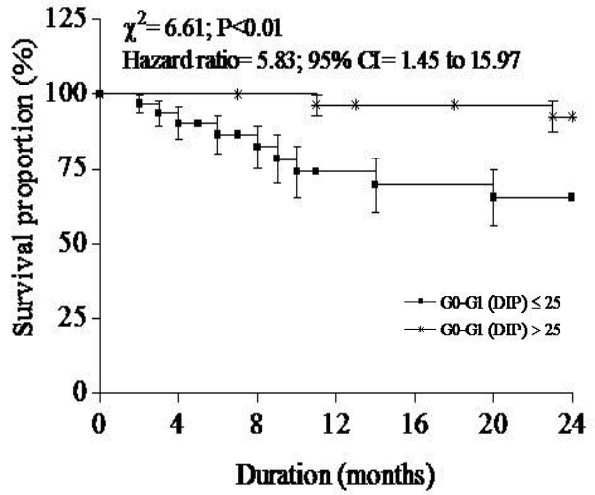

Figure 3. Comparison of 2-year overall survival proportions (%) in patients (n = 62) between G0-G1 (DIP) ≤ 25 and > 25 with 95% CI (vertical bar). The horizontal line with dots shows cumulative survival in patients having G0-G1 (DIP) ≤ 25 and horizontal line with stars shows survival of patients having G0-G1 (DIP) > 25. Star and dot represents deaths in respective groups.

senescence (*18*). This study proves that DNA content in fresh/frozen tissues may have a screening ability as diagnostic as well as prognostic significance. G0-G1 fraction (DIP) has significantly higher prognostic value, suggesting that patients with low G0-G1 (DIP) at all stages of disease were at higher risk. Seven cell cycle parameters *viz*., Diploid, DI, S (DIP), G2/G1 (DIP), G2-M (ANP), S (ANP) and G2/G1 (ANP) were not found statistically significant for diagnostic or prognostic value in patients with or without cervical neoplasia. Although DNA aneuploidy has been detected in fixed materials from uterine cervix in previous studies (*19*), it has been suggested that DNA aneuploidy is less preferred as an indicator of malignancy (*20*) and/or has no impact on prognostication of cervical carcinoma (*21*). In contrast, several researchers have found DNA aneuploidy in cervical lesions, which focused on the DNA content of fresh material from tumors (*12*) and a high S-phase fraction has been associated with poor prognosis (*5*). In this study, the prognostic value of Total S phase was also not found to be significant. Differential results in previous studies might be due to different preparation, staining and measuring techniques; different quality standards and lack of sophisticated computer software. Several previous studies reported an increased occurrence of aneuploid tumors in patients with an advanced stage of cancer (*4*). Cervical lesions with an abnormal DNA ploidy profile are more likely to persist or progress than normal diploid. In early cervical carcinomas, it has been shown that aneuploidy correlates with a high frequency of lymph node metastasis and tumor recurrences, irrespective of treatment with radiotherapy or surgery (*22*). Therefore, our results reinforce the hypothesis that DNA ploidy may be associated with progression of cervical carcinoma particularly with survival of patients. Wilailak *et al.* (*5*) concluded that patients with DNA diploid tumors have a better survival rate when compared to patients with aneuploid tumors. This study also concluded the same in terms of G0-G1 (DIP). Many investigators have reported that an abnormal DNA index is specific for malignancy, particularly in solid tumors (*23*) and statistically higher recurrence rates are seen in tumors with aneuploidy (DI > 1.5) in each of the stages IB-III of cervical cancer. Lai *et al*. (*24*) have shown that DI (DNA index) alone has promising prognostic value. However, the present study did not find DI a prognostic variable as reported in previous studies. The drawback of this study is that we could not get cervical tissue of patients during or at the end of treatment. Hence changes in cell cycle parameters during radiation treatment could not be evaluated. The prognostic evaluation of patients was done by correlating patient survival time with pre-clinical, histopatholigical examinations and cell cycle parameters. In the present study the digestion of the unfixed material was done with trypsin preferred over pepsin (*25*) and then fixed with ethanol and stained with propidium iodide. RNase was not used thus the broader CV might be due to contamination of RNA or it might be also because of particle variations *i.e*., if the samples were from the general population they might have different unknown disorders. The broadening of CV could also be because of preparation of single cell suspensions rather than single cell nuclei.

The present study concluded that G0-G1 (Diploid) may help in the diagnosis of carcinoma of the cervix which correlates well with histologically confirmed varied gradings of cervical cancer as well as patient survival. G0-G1 (Diploid) with Total S phase and Total Aneuploid fraction discriminates cases of both disease groups correctly with 100% sensitivity and specificity. Findings of this study may have clinical significance for false positive results. In future, it is suggested that if this study could be done on unknown smear samples, the threshold value of G0/G1 might result in early diagnosis of the disease as well.

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Original Article

Isolation of mesenchymal stem cells from bone marrow wastes of spinal fusion procedure (TLIF) for low back pain patients and preparation of bone dusts for transplantable autologous bone graft with a serum glue

Tomoko Ichiyanagi[1], Kouki Anabuki[2], Yoji Nishijima[3], Hirohisa Ono[3,*]

[1] *National Institute of Genetics, Mishima, Shizuoka, Japan;*
[2] *Department of Orthopedic Surgery, Nishijima Hospital, Numazu, Shizuoka, Japan;*
[3] *Department of Neurosurgery, Nishijima Hospital, Numazu, Shizuoka, Japan.*

Summary **Low back pain and subsequent disabilities are common. A lumbar spinal fusion procedure is an effective treatment with autologous bone grafts, but harvesting the bone from the iliac crest is associated with risks of complications. New treatments using stem cells together with osteoconductive and otesoinductive materials have made the procedure safer, but the inconsistency of the amount of stem cells harvested from bone marrow aspirate still remains to be solved. This study reports that the bone dusts, usually discarded as surgical wastes during transforaminal lumbar interbody fusion procedure (TLIF procedure), yielded cells which had the characteristics of mesenchymal stem cells (MSCs) *in vitro*. The cells were positive for the MSC markers and were able to differentiate in osteogenic and adipogenic directions. The cells grew robustly in an osteoconductive material, Bolheal (serum glue), and also proliferated well in culture medium supplemented with autologous serum. Therefore, the bone dust is a good candidate for the alternative source of stem cells other than bone marrow aspirate to increase the safety of the TLIF procedure.**

Keywords: Mesenchymal stem cells, bone dusts, TLIF procedure, serum glue, xenogenous material-free preparation

1. Introduction

More than 80 percent of Japanese men and women complained of low back pain in 2004 and the prevalence further increased, according to a Japanese national survey (*1*). Approximately 15 to 20% of these people suffered and experienced interference with daily activities (*2*) and it has been a major social burden with enormous cost and labor for taking care of these patients. In the U.S., the cost has been estimated to be more than $100 billion a year and is still increasing (*3-6*). It became clear that one of the major sources of the increasing cost was due to spinal fusion procedure, which is more prevalent in older populations (*4*). The spinal fusion surgery is a particular surgical procedure aimed to stabilize painful spine by connecting abnormally unstable parts of the spine, which is caused by degeneration or aging, with new bone growth by inserting bone fragments (autologous bone grafts or allografts). Recently, synthetic materials have been used although these materials themselves cannot grow the new bone but rather, attract bone forming cells, osteoblasts, on them and participate in bone growth in the surgical area as scaffolding materials (*7*,*8*). The number of fusion procedures in the lumbar region, such as transforaminal lumbar interbody fusion procedure (TLIF procedure) rose more rapidly than other fusion procedures (*4*).

The bone tissue takes much longer time to heal or to complete fusion than the soft tissue does. During the early era of the bone fusion procedures, it was a common practice to keep a patient postoperatively in an uncomfortable body cast immobilized in the bed until the bone fusion is completed in about 12 weeks after surgery, which was the estimated period required for the fusion. Recent technical and instrumental advancements

**Address correspondence to:*
Dr. Hirohisa Ono, Department Neurosurgery, Nishijima Hospital, 2835-7 O-oka, Numazu, Shizuoka 410-0022, Japan.
e-mail: hirohisa.ono@hotmail.co.jp

made the immobilization and use of the cast obsolete. The postoperative patients are much more comfortable and can be up and about the next day after the surgery as the results of intraoperative use of stabilizing metal screws (pedicle screws) and cages. However, these significant advancements in techniques and materials which provided an instant stabilization of the surgical area had not shorten the time required for the bone healing, or improved the fusion rate, and therefore the old fashioned autologous bone graft still remains as the best fusion material. The bone healing requires three fundamental factors, osteoconduction, osteoinduction, and osteogenic cells (*9*) and the autologous bone graft is the only material that processes these factors in one peace.

However, harvesting the autologous bone grafts, usually from patient's own hipbone, or the iliac crest, is well known to be associated with very high risks and surgical complications. Approximately 30% of patients developed donor site morbidity (*10*).

Despite of a complete relief of preoperative clinical symptoms from the fusion surgery, these patients suffered new symptoms after surgery such as pain and numbness in the iliac crest area, which can be severe enough to cause problems in ambulation (*11*). Other complications from harvesting autograft bone from iliac crest include wound dehiscence, infection, blood clot formation, and fracture of iliac crest with prolonged debilitating pain (*12,13*).

Growing concerns on these risks and complications for autograft harvesting have prompted investigations on the other sources for the bone fusion and the mesenchymal stem cells have recently attracted researchers as the source of osteogenic cells. A major source of the stem cells, including the mesenchymal stem cells has been bone marrow blood cells obtained by aspiration of the iliac crest or the sternum (*14*). The aspiration is simpler than surgical harvesting of the bone tissue. However, the bone marrow aspiration can be associated with serious complications such as nerve, arterial and ureteral injury, herniation of abdominal contents, sacroiliac joint instability, pelvic fracture, hematoma and infection (*15*). The aspirant has to be less than 2 cc. at a site in order to avoid contamination by peripheral blood, yet a bone marrow aspirant processing device, such as Bone Marrow Aspirate Concentrate (BMAC) system (Harvest Technologies Corporation), requires a large quantity of the aspirant to collect enough amount of cells for a graft. The system collects mononuclear cells from the bone marrow aspirant where the stem cells reside, but the number of the cells obtained remains inconsistent due to the technical difficulties.

In order to substitute the role of autologous bone graft by the stem cells, additional factor(s) have to be considered, because stem cells alone at the fusion site failed to produce meaningful bone fusion (16-*18*). Some factors for osteoconduction and osteoinduction other than the stem cells are thought to be required. For the osteoconductive material, many biological and synthetic carriers for the stem cells have been examined (*19*), and the endeavor for finding osteoinductive element(s) resulted in finding of bone morphogenetic protein (BMP)-2 (*20,21*) which is currently commercially available. However, since the source and the harvesting method are inconsistent, the use of stem cells has not exceeded the fusion rate or the surgical results of the traditional autologous bone graft operation.

Our current study is aimed to find a more consistent and safe source for the autologous bone graft and refinement of the harvesting technique during lumbar spine fusion surgery, TLIF. First, *in vitro* isolation and expansion of the stem cells from the new source was attempted, and then the behavior of the isolated stem cells in an osteoconductive material, Bolheal (a serum glue) was tested. An attempt to remove all xenogenic materials from the processing of the graft for transplantable grade was also performed by culturing the stem cells by autologous serum supplement. This study is a first step towards our final goal to establish a fusion surgery with more affordable cost and to improve safety and efficiency of the spine fusion procedure.

2. Materials and Methods

2.1. *Harvesting bone marrow wastes*

The bone marrow wastes were harvested from 10 patients who underwent spinal surgery (TLIF procedure) with pedicle screws and a cage insertion in the disc space in the lumbar area. The bone marrow wastes, consisted of blood clot and bone dusts, were usually discarded as a waste immediately to clean the surgical field. However, the wastes were collected for this study according to the method approved by the Nishijima Hospital (Numazu City, Japan) Ethics Committee after informed consent process. In brief, a regular 5 or 6 mm drill (depending upon the size of the pedicles) for the TLIF procedure was inserted into the center of the bone marrow of designated lumbar spine vertebral bodies through the pedicles of the vertebral body at the planned surgical site. This step is an essential part of the regular technique for the TLIF procedure to prepare for the stabilizer screw insertion. When the drill was removed, the attached blood clot and bone dusts were immediately washed off in heparinized saline solution. If a first attempt with 5 or 6 mm size drill yielded less bone dusts, then a 4.5 mm drill was re-inserted into the same hole for removing the blood clots in the hole (Figure 1, *right*). The remaining blood clots were also collected from the drilled hole by suction since these usually contained bone dusts fallen off the drill during withdrawal of the drill. The free flowing blood, which is thought to be contaminated by the peripheral blood,

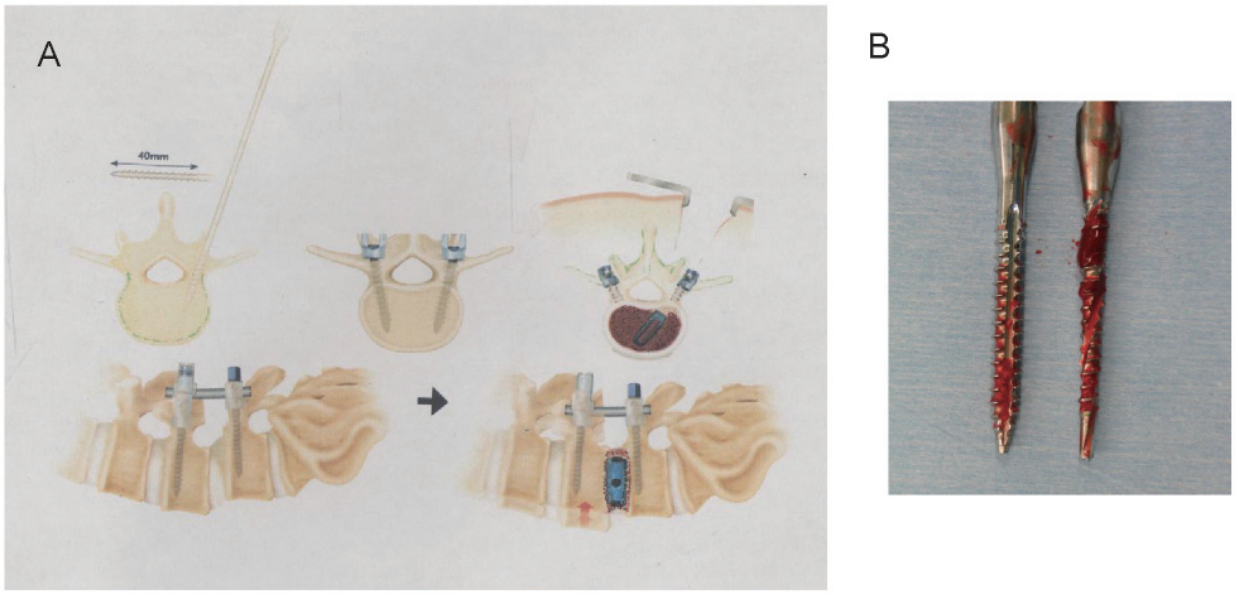

Figure 1. TLIF (transforminal lumbar interbody fusion) procedure and drills for the two-drill technique. Left: A regular TLIF drill is inserted into the center of the bone marrow and the bone dusts attached to it were harvested after making guide holes for pedicle screws. Then, a cage is inserted in the space between two vertebral bodies, and anterior sliding of one of the two unstable vertebral bodies is corrected with inserted screws and the cage (Modified from Medtronic Sofamor Danek Co., Ltd. brochure illustration). **Right**: 6 mm and 4.5 mm drills used in harvesting the bone dusts.

was not collected but rather, immediate hemostasis was obtained as in the regular surgery.

2.2. *Culture of the bone marrow wastes*

The bone marrow wastes, consisted of blood clot and bone dusts were washed 3 times in Hanks Balanced Salt Solution (Gibco) and then digested with 256 U/mL of Collagenase IX (Sigma) at 37°C for 3 h. After the treatment, samples were centrifuged to separate BD cells (bone derived cells) and CR cells (collagen released cells) according to Sakaguchi *et al*. (*22*). Obtained bone fragments or cells were cultured in DMEM with 2 mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma), and 10% FBS (Gibco) or 10% autologous serum.

2.3. *Preparation of autologous serum*

Approximately 60 mL of the whole blood from the same patient who was subjected for collecting bone dusts were first centrifuged at 160 × g for 10 min at room temperature, and the upper layer were centrifuged again at 160 × g for 10 min at room temperature. The upper layer were taken as platelet-rich plasma (PRP) and centrifuged at 780 × g for 10 min, and the supernatant was pooled as platelet poor plasma (autologous serum) and the pellet (platelets) were resuspended with HBSS and stored frozen separately. Autologous serum was heat inactivated in 56°C waterbath for 30 min, and then centrifuged for 1,640 × g for 10 min at 4°C, and the supernatant was used in the culture where indicated.

2.4. *Flow cytometric analysis*

Surface molecule expression was examined by flow cytometric analyses by the following monoclonal antibodies (mAb): anti-CD14-fluorescein isothiocyanate (FITC), anti-CD19-FITC, anti-CD45, anti-CD105-PE, anti-HLA-DR-PE (eBioscience), anti-CD34-PE (Santa Cruz Biotechnology), anti-CD73-PE (BD Bioscience), anti-CD90 mAb (Serotec, Oxford, UK). FITC or PE labeled isotype control mAbs (eBioscience) were used for directly labeled mAbs, and Alexa488-labeled goat anti-mouse IgG secondary antibody (Molecular Probes) was used for anti-CD45 and anti-CD90 unlabeled mAbs. Cells were stained with mAbs on ice for 30 min, and washed twice by PBS. Secondary antibody was applied for unlabeled mAbs and incubated on ice for 30 min, then washed by PBS. Cells were resuspended in PBS, and analyzed by FACSCalibur (BD Bioscience).

2.5. *Mesenchymal lineage differentiation*

Abilities to differentiate into multiple mesenchymal lineages of expanded human mesenchymal stem cells were examined by Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems). Briefly, MSC were grown on cover glasses in 24 well plate to 50-70% confluent for osteogenic differentiation, and 100% confluent for adipogenic differentiation. Then, osteogenic differentiation was induced by adding Osteogenic Supplement (dexamthasone, ascorbate-phosphate, and β-glycerolphosphate), and adipogenic differentiation was induced by adding Adipogenic

Supplement (hydrocortisone, isobutylmethylxanthine, and indomethacin) in αMEM Basal Medium (αMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin). Fresh induction medium or αMEM Basal Medium for negative control was replaced every 3-4 days. After 3 weeks, osteogenic differentiated cells were washed by PBS and fixed by 70% ethanol on ice for 60 min and subsequently incubated with 1% Alzarin Red S (Sigma), pH 4.2 for 10 min (*23*). Adipogenic differentiated cells were fixed by 4% paraformaldehyde in PBS for 10 min and then subjected for Oil-Red O (Sigma) staining to visualize the lipid droplets in the cells. For chondrogenic differentiation, 2.5×10^5 cells were transferred in a 15 mL conical tube and cultured as a pellet after centrifugation (200 × g, 5 min) in 0.5 mL of DMEM/F-12 Basal Medium (DMEM/F-12 supplemented with ITS Supplement, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin) with or without Chondrogenic Supplement (dexamethasone, ascorbate-phosphate, proline, pyruvate, and TGF-β3). Medium was replaced with fresh medium every 2-3 days for 3 weeks. The chondrocyte pellet was then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and washed with PBS for 5 min. The pellets were frozen in O.C.T. compound (Tissue-Tek) and the sections were cut at thickness of 5-10 μm.

2.6. *hMSC proliferation in fibrin clot*

The bone dusts or grown MSC culture were put into fibrin glue, Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) to see the growth in scaffold material. Bolheal is prepared by mixing Solution A and Solution B. Solution A contains 80 mg/mL fibrinogen, fibrin-stabilizing factor XIII (75 units/mL), and aprotinin (1,000 KIE/mL) and Solution B contains thrombin (250 units) and 40 μM $CaCl_2$ as effective ingredients. In clinical practices, Solution A and B are mixed in the ratio of 1:1 in volume, but here we diluted both Solution A and B by PBS to 1/8 of original solution before mixing. The bone dusts or expanded MSC culture were first put into diluted Solution A, and then equal volume of Solution B was added to form semi rigid gel. Culture medium was added over the gel for culture. The culture was observed by phase contrast microscope (Zeiss) or confocal microscope (Zeiss, LSM700). Cells were stained with Calcein AM (Invitrogen) 5 μg/mL for 20 min at room temperature.

3. Results

3.1. *Bone dust harvesting*

Use of the routine TLIF drills consistently yielded bone dusts. Although, the amount of bone dust differed and some bone marrow tissues were mixed with a large quantity of fat tissue, the two drill techniques provided sufficient bone dusts. The amount of the bone dusts thus obtained was enough to fill an interdiscal cage, if this is to be used in actual fusion procedure.

Postoperative course of these TLIF patients who donated the bone dusts was uneventful. No patient complained of any symptoms related to the drilling and the harvesting of the bone dusts.

3.2. *MSC-like cells from the bone dusts expanded adherent to plastic plates*

We attempted to culture bone dusts collected during the TLIF procedure from 10 patients (Ages 50-73, MSC1-MSC10). First, we cultured the bone dusts after collagenase digestion, separating BD cells and CR cells as described in "Material and Methods". Since both cells expanded adherently on plastic plates and both had MSC-like phenotype, we did not separate BD and CR cells in the later cultures because the objective of our study was to obtain as many MSCs as possible for the use in spinal fusion surgeries. Some of the bone dusts were immediately cultured without collagenase treatment and they still grew in 10% FBS supplemented DMEM (data not shown). In all cases, cells adherent to plastic plates proliferated and were able to expand to make frozen stock vials.

The bone dusts were also attempted to culture by autologous serum, so that the culture will not contain materials from other species. MSC4 was first plated in 4 groups with different conditioned medium. One was DMEM medium supplemented by 10% FBS, and the other three were supplemented with autologous serum (AS) by 5, 10, and 20%. Although the attached cells grew in 5% AS, the proliferation rate was a little lower than that of 10% FBS medium while 10% AS and 20% AS culture showed almost equal to or even better proliferation (data not shown). Hence, serum concentration of the culture medium supplemented with AS was decided at 10% for the later cell culture.

The culture grown in autologous serum was morphologically quite uniform with smooth outlines while the culture in FBS seemed heterogeneous with some widely spreaded out cells (Figure 2A). There was another difference that the culture with autologous serum was much more sensitive to trypsin-EDTA than the cells grown in FBS when detaching from the plastic surface. This observation is the same phenomenon with the observation of Shahdadfar *et al.* (*24*).

To confirm that the expanded plastic adherent cells are the mesenchymal stem cells, immuno-histological staining was also performed for CD90 (Figure 2B). CD90 was positive for the cells expanded with both FBS or AS supplemented medium.

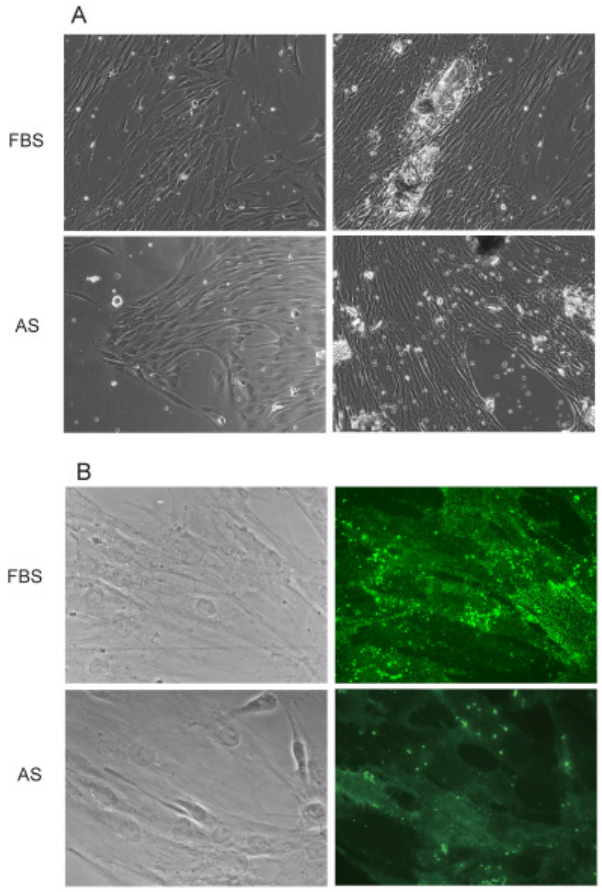

Figure 2. Morphology and immuno-histological staining of hMSC. (**A**) Phase-contrast photomicrograph of the cells expanded with 10% FBS or 10% AS (autologous serum). Original magnification, ×100. (**B**) Cells were stained by anti-CD90 mAb and Alexa 488 labeled secondary antibody. MSCs expanded in 10% FBS or 10% autologous serum supplemented medium are shown. Original magnification, ×400.

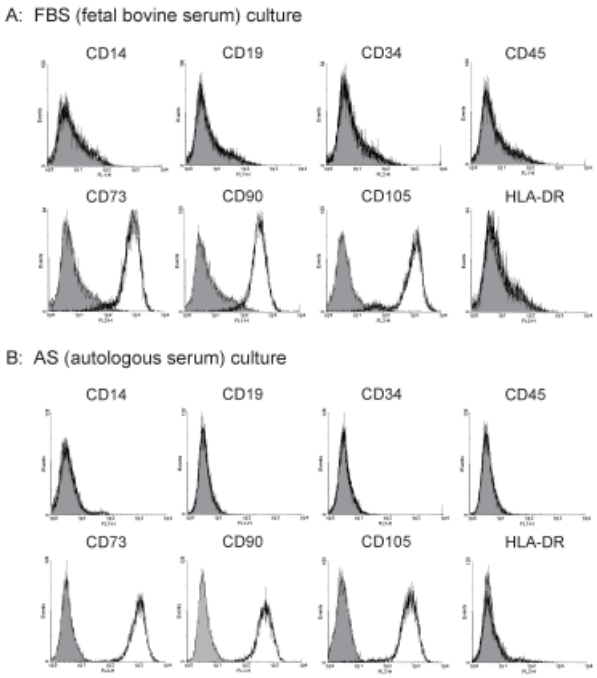

Figure 3. Phenotype of cultured human bone fragments derived mesenchymal stem cells (hMSCs). MSCs expanded in 10% FBS supplemented medium (**A**), or 10% autologous serum (**B**) were examined for their surface antigens by flow cytometric analysis. The histograms shaded in gray are negative controls, and the histograms without any color are the samples stained with respective antigens.

3.3. *Immunophenotypes of the MSC-like cells*

Cell surface markers of the expanded cells were examined by flow cytometric analysis. Both cells that were expanded with FBS (MSC1) or autologous serum (MSC4) had the same phenotype for the antigens examined. More than 95% were positive for CD73, CD90, and CD105, which showed separated fluorescence peak from the negative controls. On the other hand, the cells were less than 2% positive for CD14, CD19, CD34, CD45, HLA-DR, in which the stained fluorescence peaks completely overlapped with negative controls (Figures 3A and 3B). These phenotypes represent mesenchymal stem cells (MSCs) (*25*), and these results strongly suggest that the proliferated plastic adherent cells are indeed hMSCs. The difference between the supplemented serum in culture medium was also seen here, similarly to the optical observation, that the range of forward scatter (FS) and the side scatter (SS) of FBS culture was wider than AS culture (data not shown). This indicates the FBS cultured cells vary in their size and the state of intracellular granules and nucleus, compared to the AS cultured cells. Separating BD (bone derived) cells and CR (collagenase released) cells in preparation did not show any difference in the characteristics of the surface markers of which indicate the cells to be MSCs.

3.4. *MSC-like cells had the abilities to differentiate*

To fulfill the minimal criteria for MSC, ability to differentiate into multiple mesenchymal lineages of expanded human mesenchymal stem cells were examined for MSC1 (FBS culture) and MSC4 (AS culture) utilizing Human Mesenchymal Stem Cell Functional Identification kit (R&D Systems).

Osteogenic differentiation was induced by adding osteogenic supplement in 10% FBS + αMEM medium for 3 weeks. The cells were fixed by ice-cold ethanol and stained with Alizarin Red S to indicate the calcium accumulation. The red staining of calcium were seen in both MSC1 and MSC4, but the amount differed in a great extent (Figure 4A) and the negative controls cultured without differentiation supplement showed no staining at all (data not shown). The cells expanded in FBS medium showed the calcium accumulated staining to cover most of the cells after 3 weeks (about 80%) in differentiation medium (Figure 4A, *left*). On the other hand, the cells expanded in AS medium showed very little staining (about 10-20%) after 3 weeks of

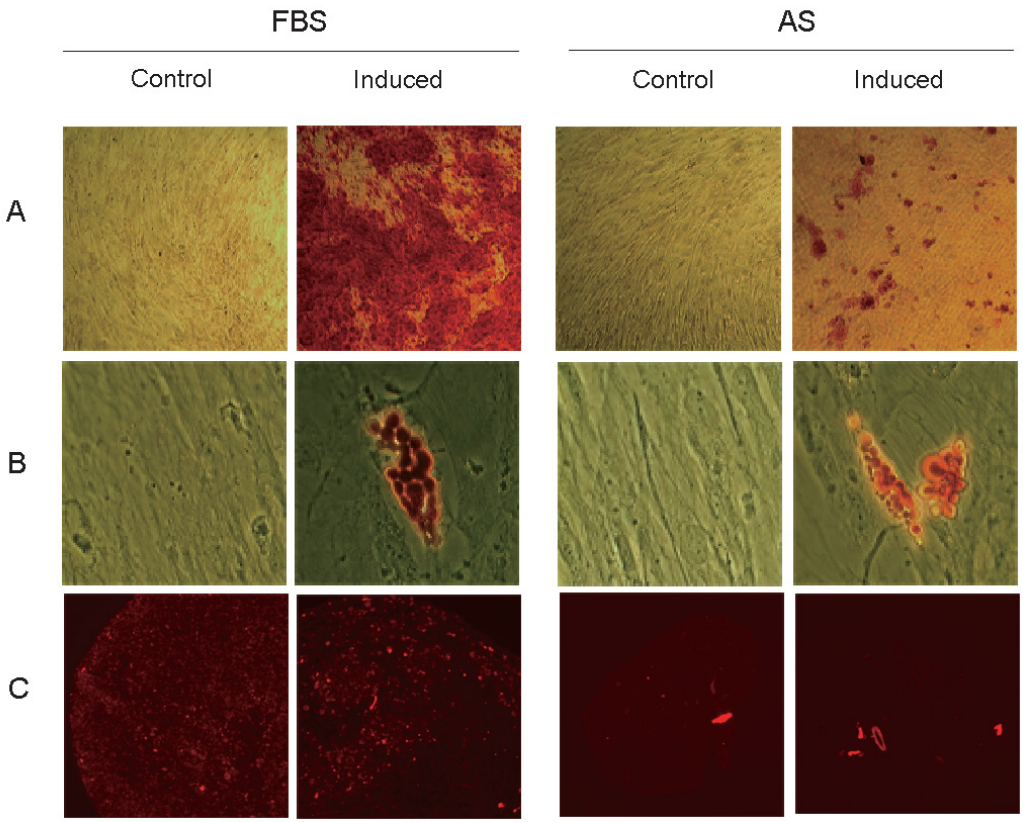

Figure 4. Differentiation ability of hMSCs. hMSCs induced to differentiate in osteogenic direction (**A**) were stained with Alizarin Red S, in adipogenic direction (**B**) were stained with Oil-red O. Original magnification, ×40, ×100, respectively. The cells induced in chondrogenic direction (**C**) were immuno-histochemically stained by anti-human aggrecan antibody as a primary antibody. Original magnification, ×100.

differentiation medium culture (Figure 4A, *right*), but the accumulation did increase after 2 more weeks of culture (data not shown). These results imply AS culture differentiate more slowly than FBS culture and do not jeopardize the ability to differentiate.

To induce differentiation in adipogenic direction, semi-confluent cells were cultured with or without adipogenic supplement in 10% FBS + αMEM medium for 3 weeks. The cells were then fixed and stained with Oil Red O, which stained the lipid vacuoles within the cells. As shown in Figure 4B, both MSC1 and MSC4 showed accumulation of lipid vacuoles within the cells that apparently indicate the cells have differentiated to adipocytes. These vacuoles were absent in negative controls (data not shown). The rate of differentiation was roughly 10-50% and was not stable in each experiment in which may be due to several culture conditions such as the freshness of the differentiation supplements and the passage number of the MSC.

Chondrogenic differentiation was induced in 15 mL conical tubes in a form of pelleted micromass. Although the number of cells at start was the same, the size of the pellets differed between MSC1 (FBS culture) and MSC4 (AS culture) that MSC4 formed much smaller mass. The pellets were examined after 3 weeks of culture by immuno-cytochemistry of human aggrecan, or collagen II to show the characteristic of chondrocyte. Despite the results of osteogenic and adipogenic differentiation, chondrogenic differentiation was not clear. MSC1 (FBS culture) showed human aggrecan (Figure 4C) or collagen II staining (data not shown) in both culture with or without differentiation supplements. The pattern of the staining was different in which the culture without the supplements showed overall dim staining while the culture with the supplements had some bright positive staining section and dark negative part (Figure 4C, *left*). On the other hand, MSC4 (AS culture) did not show much expression of aggrecan or collagen II, in both cells cultured with or without the differentiation supplements (Figure 4C, *right*).

3.5. *MSCs proliferate and migrate well in fibrin clots*

To explore the potential of hMSC in clinical use, expanded cells or bone fragments were cultured in fibrin clots, Bolheal. Instead of using Bolheal as it is prepared for fibrin glue in clinical use, we diluted fibrinogen and thrombin to make more soft gel for culturing the bone dusts or MSCs so that the cells will have enough space to grow and migrate. The

concentration of fibrin was decided based on the studies previously reported (*26*,*27*).

From a bone fragment buried inside Bolheal, growing cells were seen after 12 days of culture. Cells grew outward from the bone fragment in a bundle (Figure 5A) and gradually made a swirl like pattern inside Bolheal. It took about 4-5 weeks for cells grown from a bone fragment to fill the Bolheal gel (100 μL in volume) and expanded cells finally started to appear outside the fibrin clot (Figure 5B).

4. Discussion

Our study showed that the bone dust-derived cells cultured in FBS and also in autologous serum were plastic-adherent and expressed CD73, CD90, CD105, and were negative for CD14, CD19, CD34, CD45 and HLA-DR. In addition, these cells differentiated to osteogenic and adipogenic direction. These evidences strongly suggest that the obtained cells are mesenchymal stem cells, and the bone dusts, the waste produced during the TLIF procedure, could be a practical candidate for the source of MSCs. This report is the first to show that the possibility of using the bone dusts as the source of MSCs. We have also shown that these cells grew well in clinically available serum glue (Bolheal), suggesting it as a possible scaffold to utilize the bone dusts or expanded MSCs as autologous bone grafts.

In the aspect of the future application, this study demonstrated the possibility of using the bone dusts as the source of stem cells in TLIF procedure. Harvesting bone dusts from bone marrow was simple and safe because the process in which provided them was a routine part of TLIF surgery, and caused no complication or postoperative discomfort to the patients. The bone dusts are immediately available during surgery for the transplant as a source of stem cells, without multi-step treatment that is required for bone marrow aspirant. The TLIF surgeons prefer shorter surgical time and the immediate availability of the graft material is an important factor for adopting the techniques. The harvesting and processing of the bone dusts in current technique should satisfy the TLIF surgeons with these regards.

This study also demonstrated that the bone dust-derived cells proliferated well in the Bolheal. We need to further examine that the cells proliferated in Bolheal are also MSCs, and then the ability to differentiate in osteogenic direction within Bolheal. Then, *in vivo*

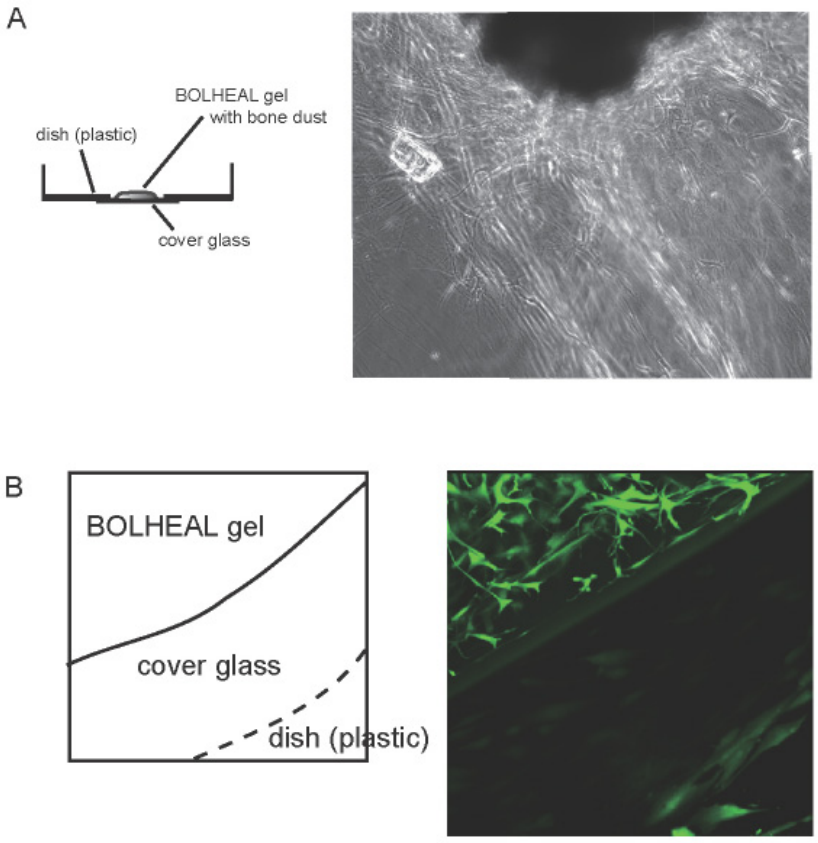

Figure 5. hMSCs culture from the bone fragments in a fibrin clot. Bone fragment was cultured in Bolheal gel with 10% FBS + DMEM medium. (**A**) Cells growing outward from the bone fragment on day 14. (**B**) Bolheal gel culture was stained with Calcein AM and observed by confocal microscope on day 59. Grown cells have come out from Bolheal onto cover glass and further grown to the dish area.

studies in experimental animals are needed to confirm the ability of the graft, Bolheal and the bone dusts or cultured MSCs, to differentiate in osteogenic direction in physiological environment. Our data indicate that the differentiation of AS cultured cells to osteogenic direction was slower or less efficient than FBS cultured cells. This observation needs to be studied more carefully and precisely, on the assumption that xenogenic materials have to be eliminated.

Appropriate osteoinductive materials are also required to be evaluated by not only the physiological effects but also the cost of the material(s). We are considering Vitamin D3 (*28-31*), Resveratrol (*32*), or 17-beta estradiol (*33*) as alternatives for expensive BMP-2.

The yield of stem cells from bone marrow aspirant is known to be inconsistent. The number of mononuclear cells harvested from the bone marrow aspirant in which the stem cells reside has been reported to be 1-2.9% of the collected cells by magnetic devices (*34*) and estimated to be 0.01-0.0001% by a density gradient preparation (*35*). Initial large cell population is a prerequisite for the survival of the transplanted cells. Therefore, keeping the bone dusts in Bolheal provides not only an appropriate scaffold for cell growth but also a safe harbor for survival. In addition, Bolheal can be mixed with osteoinductive materials for the stem cell to differentiate to bone producing cells, the osteoblast cells.

The other aim of current study was to prepare the bone dust-derived stem cells for transplantable grade by eliminating xenogenic material. Firstly, the bone dusts were cultured in autologous serum at concentration of 5, 10, and 20%. The cells proliferated in all of the three concentrations, although the proliferation rate was lower in the medium with 5% autologous serum. The elimination of collagenase treatment of the bone dusts is also in favor of keeping the bone graft non-xenogenic and since we assume to use the bone dusts immediately during the spine fusion surgery in the future application, we also attempted to culture some bone dusts without collagenase digestion. Plastic adherent cells expanded well in the same manner as the collagenase digested bone dusts did, and the characterization of these cells has to be examined. Although the serum glue, Bolheal, is known to be minimally immunogenic, immunological reaction against the serum glue can still be significant (*36,37*). In order to make it completely xenogenic, and allogenic material-free, purified thrombin could be replaced with chemically synthesized material, such as thrombin receptor activating peptide (TRAP) (*38*).

In this study, we have shown a possibility of making another protocol for the TLIF procedure in which Bolheal will be the osteoconductive material and the bone dusts from bone marrow waste for the source of stem cells.

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Original Article

Bone marrow-derived stromal cells can express neuronal markers by DHA/GPR40 signaling

Desislav B. Kaplamadzhiev[1], Hiroko Hisha[2], Yasushi Adachi[2], Susumu Ikehara[2], Anton B. Tonchev[3], Nadezhda B. Boneva[1], Ilia V. Pyko[1], Mitsuru Kikuchi[4], Masa-aki Nakaya[5], Tomohiko Wakayama[5], Shoichi Iseki[5], Tetsumori Yamashima[1,*]

[1] *Department of Restorative Neurosurgery, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan;*
[2] *First Department of Pathology, Kansai Medical University, Osaka, Japan;*
[3] *Laboratory of Cell Biology, Medical University-Varna, Varna, Bulgaria;*
[4] *Departments of Psychiatry and Neurobiology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan;*
[5] *Departments of Histology and Embryology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan.*

Summary **The exact origin of neural stem cells in the adult neurogenesis niche remains unknown. Our previous studies, however, indicated an implication of both bone marrow cells as potential progenitors of hippocampal newborn neurons and polyunsaturated fatty acids as ligands of G protein-coupled receptor 40 (GPR40) signaling. Here, we aimed at studying whether bone marrow-derived stromal cells (BMSC) treated by docosahexaenoic acid (DHA) can express neuronal markers *in vitro*. We focused on implication of DHA/GPR40 signaling for the expression of neural markers in clonally-expanded BMSC from young macaque monkeys. Cell cycle analysis revealed that the DHA plus bFGF treatment induced a decrease of BMSC proliferation and increased the cells in the G_0 resting phase. The transitions from nestin-positive progenitors *via* immature neuronal (β III-tubulin-positive) to mature neuronal (NF-M and Map2-positive) phenotypes were examined using RT-PCR, Western blot and immunocytochemistry. We detected a significant increase of GPR40 mRNA and protein expression after bFGF induction, being compared with the untreated BMSC. Addition of DHA, a representative GPR40 ligand, led to a significant down-regulation of GPR40, *i.e.,* G protein-coupled receptor-specific internalization, with a subsequent upregulation of neuronal markers such as β III-tubulin, NF-M and Map2. These data altogether suggest that adult primate BMSC can express neuronal markers with the aid of DHA/GPR40 signaling.**

Keywords: Bone marrow-derived stromal cells (BMSC), docosahexaenoic acid (DHA), basic fibroblast growth factor (bFGF), G protein-coupled receptor 40 (GPR40), monkey

1. Introduction

The G protein-coupled receptor 40 (GPR40) gene was first identified in the pancreas and brain of humans in 2003 (*1*,*2*). Subsequently, polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid were found to be endogenous ligands of GPR40 (*1*-*3*). The implications of this receptor for the insulin secretion in the pancreas have been extensively studied. However, except for ours (*4*-*6*), there are no reports studying the role of GPR40 in the brain, even though this receptor is widely distributed in the central nervous system (CNS) (*1*). Recently, Ma *et al*. found upregulation of the GPR40 expression in the primate neural stem cell niche after transient global ischemia (*4*). Furthermore, using rat neural stem cells devoid of GPR40, they confirmed that the DHA-induced neuronal differentiation of these cells occur after transfection of GPR40 gene (*6*). These findings reinforced a putative involvement of PUFA/GPR40 signaling in the neurogenic niche of the adult

**Address correspondence to:*
Dr. Tetsumori Yamashima, Department of Restorative Neurosurgery, Kanazawa University Graduate School of Medical Science, Takara-machi 13-1, Kanazawa 920-8641, Japan.
e-mail: yamashim@med.kanazawa-u.ac.jp

brain (*5*).

Biological effects of PUFA, particularly DHA, are thought to include incorporation into cell membrane phospholipids thus preserving membrane fluidity, its modulation of DNA transcription, or its effect on the synthesis of ATP in the mitochondria. However, it is also probable that DHA may serve as a secreted ligand that can bind with the GPR40 receptor and elicit intracellular signaling for the regulation of adult neurogenesis (*5*). There are several lines of evidence supporting this possibility. First, GPR40 couples mainly with the G protein α-subunit of the Gq family resulting in elevation of intracellular Ca^{2+} and stimulation of protein kinase C (PKC) activity (*1,2,6*). Second, after transfection of GPR40 into PC12 cells, they show a transient Ca^{2+} mobilization in response to arachidonic acid (*5*) which is functionally similar to DHA, regarding its ability to induce neuronal differentiation (*7*). Third, accumulating data indicate involvement of DHA in the differentiation of neural stem/progenitor cells (NSC) (*6,8-10*), embryonic stem (ES) cells (*11*), and bone marrow-derived mesenchymal stem cells (BM-MSC) (*7*). Moreover, DHA contributes to survival and neurite outgrowth in the primary culture of cortical neurons (*12*), hippocampal neurons (*9*), and ES-derived neuronal cells (*11*). Finally, data from clinical studies demonstrate improvement of visual acuity (*13*) and cognitive ability (*14*) in infants who were given a formula supplemented with DHA plus arachidonic acid or DHA alone. At the same time, a deficiency of DHA results in a poor performance on cognitive and behavioral tests, while the supplementation with DHA recovers learning ability and memory-related performance in experimental animals (*15*) as well as in human patients with memory impairment (*5*).

The adult hippocampus of mammals, including primates, retains regeneration capability from neural stem cells generating granule neurons in the dentate gyrus. The exact origin of these progenitors has been a subject of extensive research, however, there is no worldwide consensus about this. It has been previously suggested that the vascular adventitia is a potential precursor cell source in the neurogenic niche of adult monkey hippocampus (*16*). This suggestion has been subsequently supported by the discovery of cells that were enriched in markers of neural tissue-committed stem cells residing in bone marrow and mobilized "on demand" into the peripheral blood following stroke (*17*). It is now accepted that such stem cells and the above-mentioned perivascular neural progenitors (*16*) may share a common origin, being regarded as *in situ* counterparts of bone marrow stroma (*18*). Notably, endogenous hipocampal progenitors as well as bone marrow stromal cells express GPR40 (*1,4,19*). The changes in the brain PUFA composition of rats with cerebral ischemia following transplantation of BM-MSC (*20*) further support such possibility that the postischemic enhancement of adult neurogenesis might be PUFA-dependent presumably *via* GPR40 activation. Based on these data, we designed an experimental paradigm, in which the expression of GPR40 and a set of neuronal markers such as nestin, β III-tubulin, NF-M, and Map2 were examined in clonally-expanded BMSC. BMSC were induced to express neuronal phenotype by bFGF as previously described (*21*), and the effect of DHA, a representative GPR40 receptor ligand (*1-3*) was evaluated.

2. Materials and Methods

2.1. *Harvest of monkey bone marrow cells (BMC)*

Animal experiments were performed at the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center. Surgical procedures and postoperative care of animals were in accordance with its guidelines and those of the National Institutes of Health for Care and Use of Primates. Three infant monkeys (*Macaca fuscata*) weighing 2-3 kg supplied by National Bioresource Project (Okazaki, Japan), were used for the BMC harvest. The contamination of the collected BMC with peripheral blood was prevented (*22*). Under general anesthesia with 1-1.5% fluothane mixed with 33% O_2 and 67% N_2O, a skin incision of 10 cm was made and the femoral bone surface was disclosed. Two aspiration needles (Angiotech, DBMNI 1601 - 16G) were inserted into the bone marrow cavity at proximal and distal span. The BMC samples were collected in a sterile 50 mL Falcon tube containing 30 mL Dulbecco's phosphate buffered saline (DPBS) and 2500E heparin sodium salt (Sigma-Aldrich, H3149) by perfusion.

2.2. *Experimental groups*

Clonally-derived BMSC were cultured in the neurobasal media with N-2 supplement (R&D Systems, AR003) without serum. The specific reagents of interest were added to the medium as adopted from previous experiments of *in vitro* differentiation of NSC or neuronal induction of multipotent stromal cells from bone marrow (*21,23-26*). Experimental groups were as follows: neuronal phenotype induction with bFGF, DHA alone without bFGF, and DHA plus bFGF.

2.3. *Expansion of BMSC*

Single-cell suspension from fresh BMC (30 mL) was layered over density gradient media (Axis-Shield, Lymphoprep, 1114544) and centrifuged for 30 min, 150 × 10 rpm at 20°C. Mononuclear fraction of whole BMC samples was collected at the interface and re-suspended at $1 \times 10^4/cm^2$ in 5 mL growth media (GM) in Cell Culture Flask, 25 cm^2 (BD Biosciences Labware, BD BioCoat, 354532). GM contained: minimum essential

medium alpha (α-MEM) with GlutaMAX (Invitrogen, GIBCO, 32571036), 10-15% fetal bovine serum (FBS) MSC-Qualified (Invitrogen, GIBCO, 12662029), and 1% antibiotic-antimycotic (Invitrogen, GIBCO, 15240062). Adherent cells were kept and after 50-60% confluence, BMSC were transferred and further expanded from single-cell colonies derived by serial dilution in 48 well plates (Corning, Cell Wells, 258301). Each well containing less than 10 adherent cells after 8 hours of incubation was further expanded. Clonal expansion of 0.5-1 × 10^3/cm^2 BMSC from single-cell derived colonies was cultured in 5 mL expansion media (EM). The medium contained the same reagents described for the GM except that the FBS concentration was lowered to 3%. For non-clonal expansion 1 × 10^4/cm^2 adherent BMSC were incubated in 5 mL GM. Cell viability was evaluated by trypan blue (0.5%, w/v in DPBS) exclusion. At least 90% viability was confirmed prior to each sub-culturing. Cumulative population doubling (CPD) was calculated by counting viable cells with a hemocytometer chamber.

2.4. *Differentiation of BMSC to adipocytes, osteoblasts and chondrocytes*

To induce adipogenic differentiation, clonally-expanded BMSC were incubated in 3 cycles with adipogenic induction/maintenance medium (Cambrex Bio Science, PT-3004) following the manufacturer's instructions. Osteogenic differentiation was induced from subconfluent clonally-expanded monkey BMSC cultured in an osteogenesis induction medium (Cambrex Bio Science, PT-3003) for 5 weeks in flasks until morphological changes could be seen. Chondrogenic differentiation was performed according to the standard protocol in chondrogenic medium (Cambrex Bio Science, PT-3003) for 5 weeks.

2.5. *FACS analyses of BMSC*

Clonally-derived populations of BMSC from expansion adherent culture were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) against non-human primate CD3, CD4, CD8, CD14, CD29, CD31, CD34, and CD45 (BD Biosciences, San Jose, CA); against human CD90 (Biotrend, Germany), CD73 (BD Pharmingen, CA, USA), and CD105 (Serotec, UK). The stained cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

2.6. *Induction of neuronal phenotype in BMSC*

0.5-1 × 10^5 clonally-expanded BMSC were cultured in 5 mL neuronal medium in 25 cm^2 flasks (BD Biosciences Labware, BD BioCoat, 354532). The medium contained: neurobasal media (Invitrogen, GIBCO, 21103049), L-alanyl-L-glutamine a dipeptide substitute for L-glutamine (Invitrogen, GIBCO, GLUTAMAX, 35050061), N-2 Plus media supplement (R&D Systems, AR003), 1% antibiotic-antimycotic (Invitrogen, GIBCO, 15240062). Neuronal commitment with bFGF (Sigma-Aldrich, Fibroblast growth factor-basic from bovine pituitary, F5392) was adopted from previous works (*21*,*26*). DHA (Sigma-Aldrich, *cis*-4,7,10,13,16,19-docosahexaenoic acid, D2534) was continuously added to the culture medium for 14 days. bFGF was used in 10 ng/mL working concentration and DHA was used as bovine serum albumin (BSA) complexes with DHA at 10 μM final concentration.

2.7. *Immunocytochemical analysis*

Cells were grown in a Lab-Tek-Chamber Slide System (Nalge Nunc International, Permanox, 177437) at the following seeding densities; 0.5-1 × 10^3/cm^2 for clonal expansion and 1 × 10^5/cm^2 for neuronal phenotype induction. These were then fixed and stained with IAbs as follows: rabbit anti-GPR40 (1:100) (*4*,*27*), mouse anti-GPR40 (1:250, TransGenic Inc., Japan, Clone No. G16, KAL-KG116), mouse anti-nestin (1:200, Chemicon, Millipore, MAB5326), mouse anti-neuronal β III-tubulin (1:500, Covance, Clone Tuj1, MMS-435P), mouse anti-neurofilament medium chain, NF-M (1:1,000, Chemicon, Millipore, MAB5254), rabbit anti-Map2 (1:1,000, Chemicon, Millipore, AB5622). Secondary antibodies (IIAb) were applied for 2 h at 1:200 dilution, in green: anti-rabbit Alexa Fluor 488 (Invitrogen, A11034), anti-mouse Alexa Fluor 488 (Invitrogen, A11001) and in red: anti-rabbit Alexa Flour 546 (Invitrogen, A11010), anti-mouse Alexa Flour 546 (Invitrogen, A11018). Digital images were acquired using a confocal laser scanning microscope (LSM 510; Carl Zeiss, Tokyo, Japan) and LSM 510 software (version 3.2 SP2, Carl Zeiss, Germany). The number of positive cells of the whole visual field (magnification 5×) was determined as a percentage of the total number of DAPI-labeled cell nuclei. In a representative experiment, a total of 300 cell nuclei positive for DAPI (100%) per 3-5 random visual fields were counted for any given expression marker.

2.8. *Western blotting*

Protein extraction was done with RIPA buffer (Sigma-Aldrich, R0278) and Protease Inhibitor Cocktail (Sigma-Aldrich, P8340); for GPR40 experiment samples 0.1% Triton-X was also used. Total protein concentration in the supernatant was estimated by Bradford assay using sample preparation reagents (Bio-Rad, 500-0005, 500-0006) and spectrophotometer (GE Healthcare, GeneQuant pro RNA/DNA Calculator, 80-2114-98). For each experiment, 20 μg total protein were added to a 20 μL solution containing 1:1 (v/v)

sample in sample buffer (4× Tris-Cl/SDS, pH 6.8, 125 mM; 20% Glycerol; 4% SDS; DTT 0.2 M; ~0.01% bromophenol blue) and denaturized at 100°C for 3 min. The electrophoresis was done in Laemmli's buffer system mini-gel (Bio Craft, BE-111) and transferred to PVDF membrane (ATTO, Japan, Clear Blot Membrane-p, AE-6667). IAb and dilutions were as follows: mouse anti-GPR40 (1:1,000, TransGenic Inc., Japan, Clone No. G16, KAL-KG116), rabbit anti-GPR40 (1:1,000) (*4,27*), mouse anti-nestin (1:1,000, Chemicon, Millipore, MAB5326), mouse anti-β III-tubulin (1:500, Sigma-Aldrich, Clone SDL.3D10, T8660), rabbit anti-Map2 (1:2,000, Chemicon, Millipore, AB5622) and mouse anti-NF-M (1:1,000, Chemicon, Millipore, MAB5254). A horseradish peroxidase-conjugated anti-rabbit or mouse IgG (1:10,000, Sigma-Aldrich) was used for visualization. A digital image of the signal from reacted proteins was acquired using an ECL Advanced Western Blot Detection Kit (GE Healthcare, PRN2135) and compact luminescent image analysis system (Fujifilm, LAS-4000mini) with LAS-4000mini Image Reader software v.2.0. Densitometric analysis was done using image processing software (NIH, ImageJ, 1.410).

2.9. *Cell cycle analysis*

BMSC were incubated with 10 μM BrdU (Sigma-Aldrich, B9285) for 2 h and immunostained with rat anti-BrdU (1:100, Harlan SERA-LAB) and mouse anti-Ki-67 (1:100, DAKO, Clone MIB-1, M7240) primary antibodies. The same protocol described in the immunocytochemistry was used, except for the inclusion of a DNA denaturation step by incubation in 2 N HCl for 30 min at 37°C. The acid was neutralized by immersing the samples in borate buffer. Assessment of the proliferation capacity was performed with laser confocal microscope at 24 and 72 h after the induction of neuronal phenotype.

2.10. *RNA extraction and RT-PCR analysis*

Total RNA from the samples was isolated using TRI Reagent (Sigma-Aldrich, T9424). Gene specific primers for semiquantitative RT-PCR analysis was generated using the NCBI Primer-BLAST software (*http://www.ncbi.nlm.nih.gov/tools/primer-blast/*) and NCBI Reference Sequences (whole genome assembly released by the Macaca mulatta Genome Sequencing Consortium as Mmul_051212, February 2006, whole genome shotgun sequence, *http://www.hgsc.bcm.tmc.edu/projects/rmacaque/*) provided by NIH online resources. Reverse transcription was done with First-Strand Synthesis System for RT-PCR (Invitrogen, SuperScript III, 18080-051) from 1 μg of total RNA with 50 μM Oligo (dT)20. PCR reaction was carried out in thermal cycler (Applied Biosystems, Gene Amp 9700) with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, 11304-011). Specific primers and conditions are shown in Table 1. Visualization of the corresponding band after electrophoresis in 2% Agarose gel (AMRESCO, Agarose HT, K449-500G) was done with ethidium bromide staining. A digital image was acquired using Luminescent Image Analysis System (Fujifilm, LAS-4000mini) with UV 2020 transilluminator set and image capture software, LAS-4000mini Image Reader v.2.0.

3. Results

3.1. *Characterization of clonally-expanded BMSC*

Clonally-expanded BMSC complied with the minimal criteria being suggested by the International Society for Cellular Therapy (*28*), and shared a certain similarity to mesenchymal stem cells in regards of expressed characteristic surface molecules. They sustained tri-lineage mesenchymal differentiation potential and extended self-renewal potential. Osteogenic induction was confirmed by von Kossa staining 4 weeks after the induction (Figure 1A). Adipogenic differentiation was confirmed by oil red O staining 5 weeks after the induction (Figure 1B). Furthermore, chondrogenic differentiation was confirmed by staining paraffin sections of the micromass with Alcian blue (Figure 1C).

Phenotype characterization of single cell-derived colonies of BMSC were obtained by flow cytometry. The cells were negative for lymphoid markers such as CD3, CD4, and CD8 (data not shown), an endothelial cell maker CD31, and hematopoietic cell markers CD14, CD34, and CD45 (Figure 1D). In contrast, BMSC were strongly positive for CD29 and CD90

Table 1. List of specific primers and amplification conditions for RT-PCR

NCBI Accession	Aliases	Sequence (5'-3')		Tm, Cycles	Size (bp)
		Forward	Reverse		
XM_001094514.1	GPR40 (FFAR1)	GGCCTCACTGTGCCCTGTCT	CAGGCCTCCAGGCAGACG	56, 30	297
XM_001116693.1	Nestin	GCAGGAGGAGTTGGGTTCTGG	TCCTCCCCCTCCTCCTCTTC	58, 30	276
XR_011045.1	βIII-Tubulin	ATCCGGACCGCATCATGAAC	ACCATGTTGACGGCCAGCTT	58, 28	298
XM_001106908.1	NEF3 (NF-M)	GCACATTTGCAGGAAGCATCAC	GCTTCTGGCTCCTCTCCCTTTT	58, 28	268
XP_001105471	GAPDH	GCACATTTGCAGGAAGCATCAC	GCTTCTGGCTCCTCTCCCTTTT	60, 22	263
XM_001109309.1	Map2	ACCGGGAGAGGCAGAATTTCCAC	AGCGCTTTTCTGGGCTCTTGGT	58, 28	319

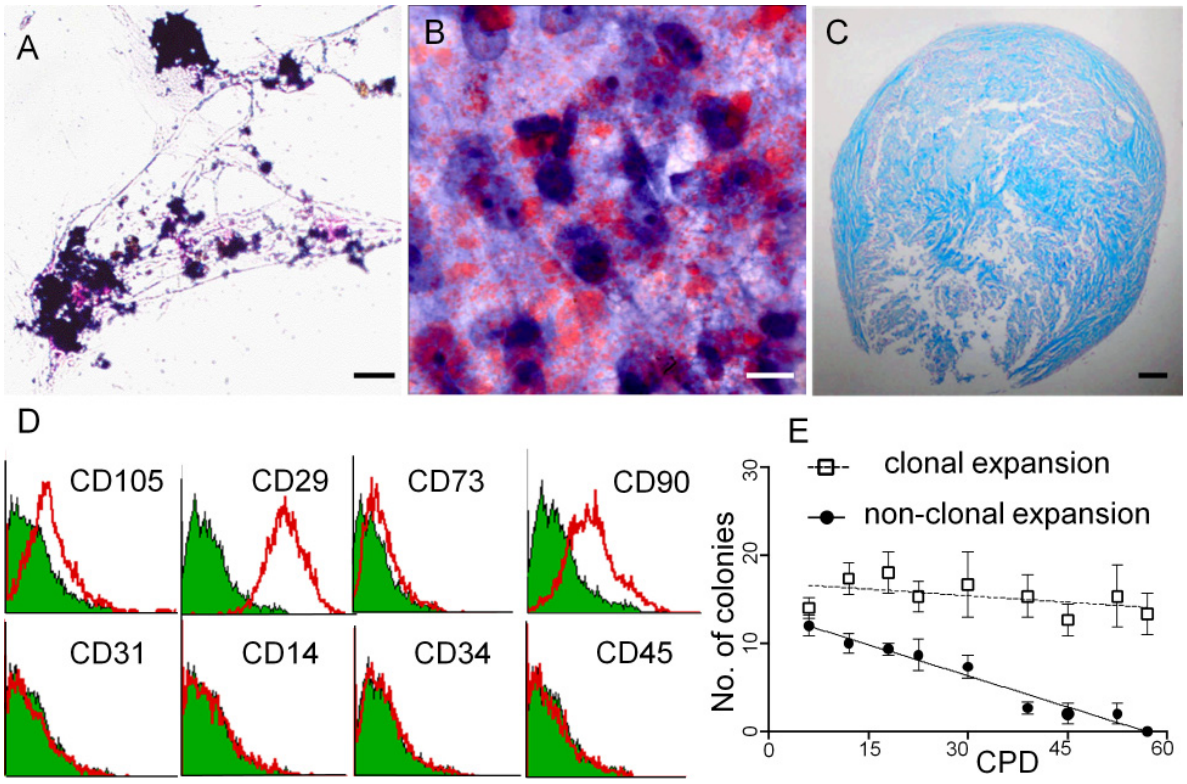

Figure 1. Characterization of bone marrow-derived stromal cells (BMSC). (**A-C**) Differentiation assay of clonally-expanded BMSC; **A**, osteogenic differentiation showing calcium deposition at 21 days of differentiation. von Kossa staining, Scale bar = 50 μm; **B**, adipogenic differentiation showing aggregates of lipid vacuoles at 35 days in adipogenic induction medium. Oil Red O staining, Scale bar = 20 μm; **C**, chondrogenic differentiation of the micromass positive for proteoglycan at 35 days in chondrogenic induction medium. Alcian blue staining, Scale bar = 100 μm. (**D**) Surface molecules in BMSC – clonally-expanded BMSC stained with a panel of antibodies reacting with hematolymphoid markers and cell adhesion molecules analyzed by FACScan. The green region indicates the cells stained with isotype-matched control. Representative staining patterns of three independent experiments. (**E**) BMSC obtained by adherence to plastic of mononuclear bone marrow cells and expanded from single cell-derived colonies denoted with open square (clonal expansion) were compared to non-clonal adherent BMSC in standard condition denoted with filled black circle (non-clonal expansion). Clonal expanssion in the low serum condition selectively favored cells with potential for extended colony formation (number of the colonies on vertical axis) and self-renewal (number of cumulative population doublings – CPD on horizontal axis). These properties in non-clonally expanded BMSC on contrary decreased with the time in culture. ($p < 0.0001$; $\alpha = 0.05$, $n = 3$, error bars represent S.E.M.)

while weakly positive for CD73 and CD105. The negative staining for CD14 and CD31 indicated that the present clonally-expanded BMSC did not contain macrophages or endothelial cells. Single cell-derived colonies of monkey BMSC expanded in the low-serum at low-seeding density, showed a consistent colony formation and an extended subculture potential. On the contrary, non-clonal cultures maintained under standard condition with 10% serum at high seeding densities showed decreased ability to form colonies and limited subculture potential (Figure 1E).

3.2. *Cell cycle analysis*

We used a thymidine analogue BrdU incorporated into the cell during the S-phase and the expression level of Ki67 protein to track down the proliferation rate in the course of the experiment. Proliferation marker Ki67 ubiquitously labels all proliferating cells. By double immunostaining we assessed the number of cells, which upon exit from cell cycle halt the production of Ki67 and retain the BrdU trait, thus $Ki67^{-}/BrdU^{+}$ phenotype indicated differentiating or apoptotic cells. Analysis of cell proliferation 24 h after the BrdU inclusion in the cell culture media by BrdU/Ki67 labeling revealed no significant difference between the used protocols (data not shown). However, 72 h after neuronal induction and BrdU inclusion (Figure 2A), we found a significant decrease of the proliferation rate represented by the number of $Ki67^{+}/BrdU^{+}$ cells in BMSC treated with bFGF and particularly in the group treated with DHA plus bFGF protocol, compared with the control (Figure 2B). Also, the number of cells exiting the cell cycle ($Ki67^{-}/BrdU^{+}$ phenotype) markedly increased during the DHA-plus-bFGF treatment (Figure 2C). We did not detect signs of cell death at the time of the cell cycle analysis as assessed by employing the PI and DAPI double-staining method (*29*) (data not shown).

3.3. *Expression of immature neural markers*

The expression of neural stem/progenitor cell or

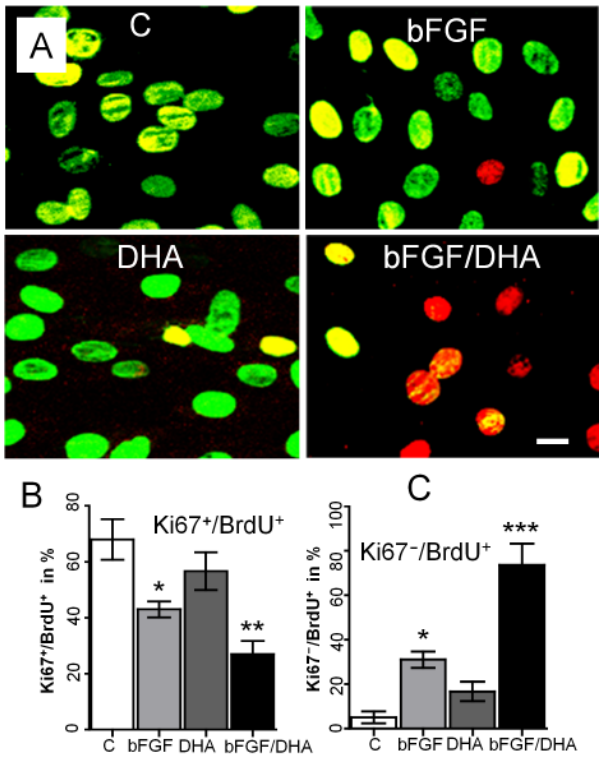

Figure 2. Cell cycle analysis of BMSC. (A) Double immunofluorescent staining for Ki67 in green channel and BrdU in red, 72 h after the treatment with the following protocols: **C**, control group (neurobasal media with N2 supplement); bFGF, treatment with basic fibroblast growth factor; DHA, treatment with docosahexaenoic acid; bFGF/DHA, combination treatment. Scale bar = 20 µm. The data, 72 h after the onset of the experiment revealed changes in the proliferation rate being most prominent in the bFGF/DHA-treated group. (**B** and **C**) Quantification of the immunoreactive BMSC after 72 h as percentage of all DAPI-positive nuclei and ANOVA statistical analysis with Dunnet post hoc test in the examined groups (n = 3, error bars represents S.E.M.). There was a statistically significant increase of the cells having quiescent non-proliferative phenotype ($BrdU^+/Ki67^-$ cells in red) as well as decreased proliferative phenotype ($BrdU^+/Ki67^+$ cells in yellow); *, $p < 0.05$ *vs.* control; **, $p < 0.01$ *vs.* control; ***, $p < 0.001$ *vs.* control.

immature neuronal markers nestin and β III-tubulin were examined by immunocytochemistry (Figures 3A-3D), RT-PCR (Figure 3E), and Western blot (Figure 3F). Both nestin and β III-tubulin were positive in the control group, although the β III-tubulin expression was considerably lower (Figures 3A, 3E, 3F, and 3G). One week after the bFGF induction, nestin showed down-regulation concomitant with a β III-tubulin increase, at both mRNA and protein levels (Figures 3B, 3E, 3F, and 3G). The immunocytochemical (Figure 3A) and RT-PCR (Figure 3E) analyses were compatible with the Western blot data (Figure 3F). DHA alone could not cause a significant change in the expression of the examined markers (Figures 3C, 3E, 3F, and 3G). However, DHA addition to the bFGF protocol markedly reduced nestin while significantly enhanced β III-tubulin expression (Figures 3D-3G).

3.4. *Expression of mature neuronal markers*

On the second week after the induction, we examined the expression of the mature neuronal markers such as NF-M and Map2. The control group of BMSC that was cultured in conventional media as well as BMSC treated with DHA alone, exhibited weak expression of mature neuronal markers. In contrast, mature neuronal markers were abundant in the groups treated with bFGF alone or with bFGF plus DHA. Notably, although both bFGF-alone and FGF-plus-DHA protocols induced NF-M and Map2 mRNA, the bFGF-alone protocol could not induce a significant Map2 protein expression (Figures 4A-4C). NF-M/Map2 double-positive cells had long processes (Figure 4D, *arrow*) and their number was significantly higher during combined bFGF-DHA treatment (Figure 4E).

3.5. *GPR40 receptor expression*

The expression level of GPR40 mRNA and protein were analyzed by RT-PCR, immunocytochemistry, and Western blot in non-treated BMSC, cultured in conventional neural media (control group; Figures 5A, 5E, and 5F), as well as in the three defined experimental conditions of bFGF alone (Figures 5B, 5E, and 5F), DHA alone (Figures 5C, 5E, and 5F), and a combination of bFGF and DHA (Figures 5D-5F). The RT-PCR results were compatible with those of Western blot. GPR40 receptor showed low expression in the control group (Figures 5E-5G) with a predominant localization at the cell membrane (Figure 5A). Interestingly, when BMSC were cultured with bFGF, GPR40 transcripts and protein were significantly upregulated (Figures 5E-5G). The immunostaining showed a cytoplasmic/cell membrane pattern (Figure 5B). In contrast, with DHA alone, GPR40 showed a significantly lower expression (Figures 5E-5G) with a scarce distribution in the cytoplasm and negligible immunofluorescent signal at the cell membrane (Figure 5C). There were no significant changes in the GPR40 expression observed when DHA was applied in addition to bFGF, as compared to the control. By the comparison between the bFGF-alone group and bFGF-DHA group, the latter showed a significant decrease in the GPR40 production (Figures 5E-5G). The immunostaining pattern of the cells in the bFGF-DHA group was similar to that observed in the DHA-alone group (Figure 5C).

4. Discussion

In the adult CNS regenerative process associated with both non-ischemic (*30,31*) and post-ischemic conditions (*32-34*), bFGF is an important *in vivo* signal for the intrinsic turnover of neural stem/progenitor cells. Similarly, *in vitro*, bFGF can induce neuronal phenotype in BM-MSC by binding to a specific bFGF

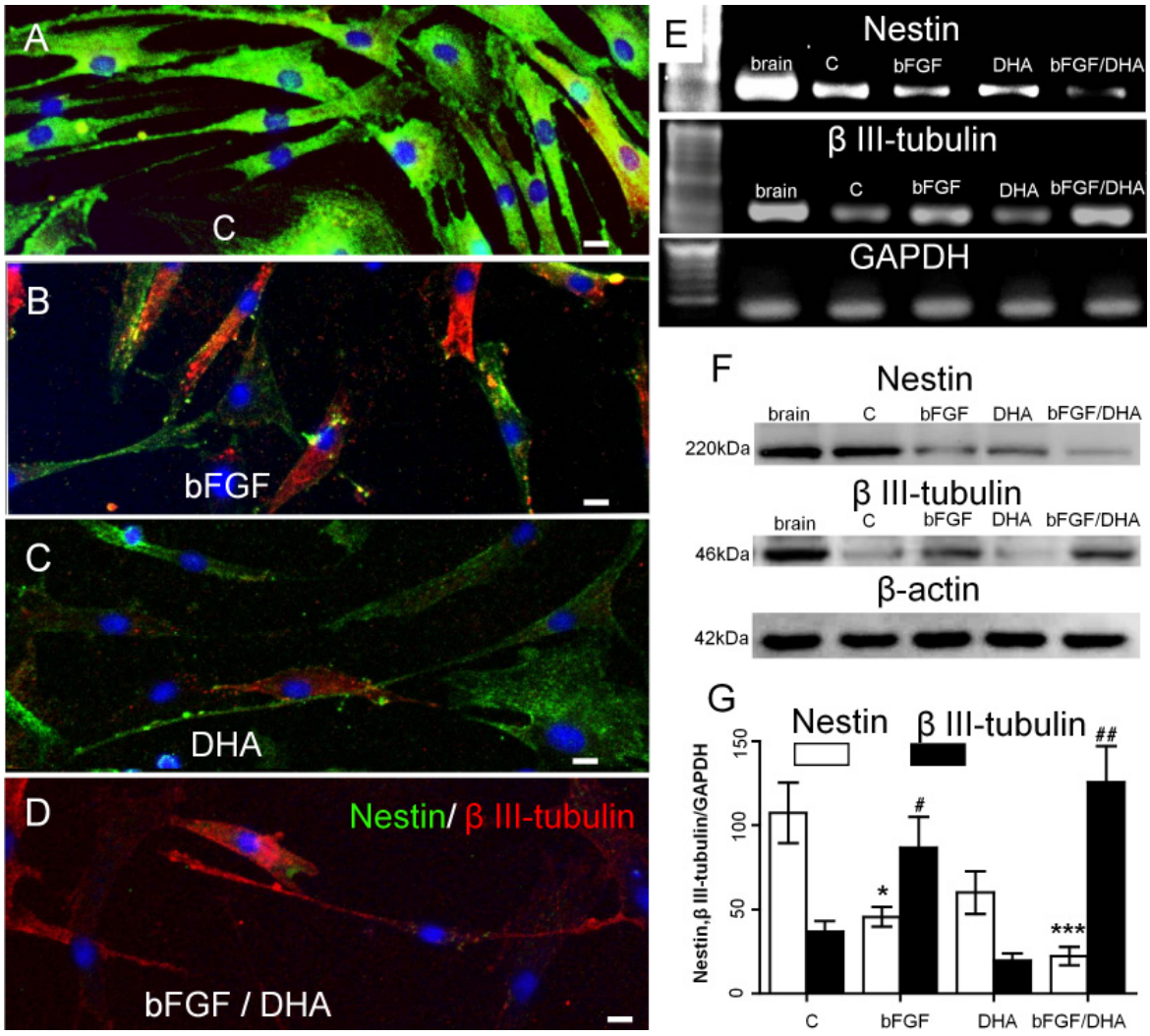

Figure 3. Expression of neural stem/progenitor marker nestin and immature neuronal marker β III-tubulin 7 days after treatment of clonally-expanded BMSC. Double immunofluorescent staining for nestin in green and β III-tubulin in red. (**A**) Control group. (**B**) bFGF treatment. (**C**) DHA treatment. (**D**) bFGF/DHA combination treatment. Scale bars = 20 μm. (**E**) RT-PCR for nestin and β III-tubulin and (**F**) corresponding immunoblots, showed similar expression level. (**G**) Quantifications and ANOVA statistical analysis ($n = 3$, error bars represents S.E.M.) of the RT-PCR results, nestin and β III-tubulin are calculated as a ratio to GAPDH. *, $p < 0.05$ *vs*. nestin, control; #, $p < 0.05$ *vs*. β III-tubulin, control; ***, $p < 0.001$ *vs*. nestin, control; ##, $p < 0.01$ *vs*. β III-tubulin, control.

receptor (*21*). The expression of nestin is widely regarded as a marker for neuronal progenitors. Being gradually replaced by filamentous proteins expressed by functionally mature neurons, nestin shows a temporal relation to the maturation towards neuronal lineage during development. In rodents, although yet controversial, nestin expression is considered also a prerequisite for the specialization of BM-MSC towards the neural lineage (*35*).

In this study, we confirmed that BMSC express nestin, and bFGF treatment decreases its expression along with the increase in expression of immature neuronal marker β III-tubulin. DHA treatment in addition to bFGF showed that BMSC can express mature neuronal markers such as NF-M and Map2. Interestingly, we found that upregulation of GPR40 receptor is triggered by bFGF. Subsequent DHA addition to the medium, however, provoked the turnover of GPR40 receptor. The enhancement of neuronal phenotype in BMSC after DHA treatment in addition to bFGF, was consistent with the data from the previous *in vitro* and *in vivo* NSC experiments where an identical relation between stem cell maturation and DHA was found in the course of their neuronal differentiation (*10*).

Although that BMSC lacked the capacity to produce mature neuronal markers being compared to the control group, when DHA was applied alone (Figure 4), there was a down-regulation of GPR40 receptor expression (Figures 5C, 5E, 5F, and 5G). The observed GPR40 down-regulation can be explained by the concept of 'receptor internalization'. Rapid desensitization and receptor trafficking are well-known to occur for tightly controlling the temporal and spatial regulation of G protein-coupled receptor (GPCR) signaling. In 1996, the β-arrestin-dependent internalization of GPCR that facilitates receptor uncoupling from G proteins, was

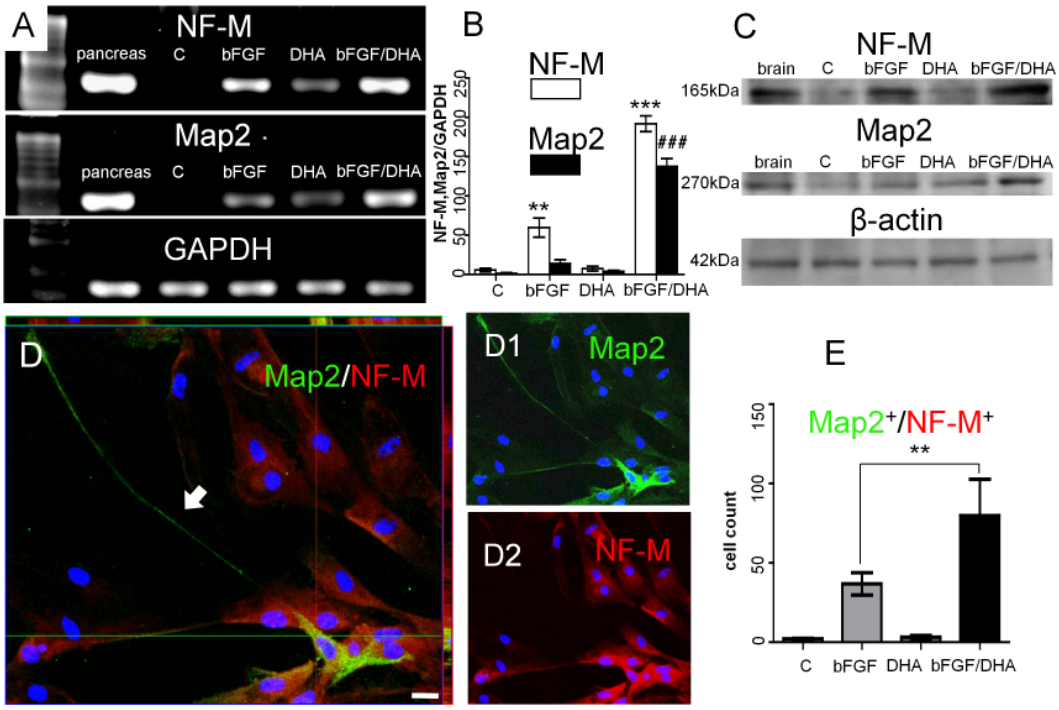

Figure 4. Expression of mature neuronal markers neurofilament medium chain (NF-M) and microtubule associated protein 2 (Map2) 14 days after treatment of clonally-expanded BMSC. (**A**) RT-PCR of NF-M and Map2 and (**C**) corresponding immunoblots, showed similar expression levels. (**B**) Quantifications and statistical analysis ($n = 3$, error bars represent S.E.M.) of the RT-PCR results subjected to ANOVA. NF-M and Map2 are calculated as ratio to GAPDH. **, $p < 0.01$, *vs.* NF-M, control; ***, $p < 0.001$, *vs.* NF-M, control; ###, $p < 0.001$ *vs.* Map2, control. (**D**) Double immunofluorescent staining of bFGF/DHA-treated BMSC; NF-M - red, Map2 - green. Scale bar = 20 μm. Map2 was positive in the process (**D**, *arrow*) and the whole cytoplasm (**D1**) while NF-M was distributed predominantly in the cytoplasm (**D2**). Some cells also showed a co-staining in the cytoplasm. (**E**) The cell number of double-positive NF-M/Map2 was significantly higher in the bFGF/DHA treatment group, compared with the bFGF group (ANOVA, Bonferroni's post-hoc between the two groups bFGF *vs.* bFGF/DHA; **, $p < 0.01$).

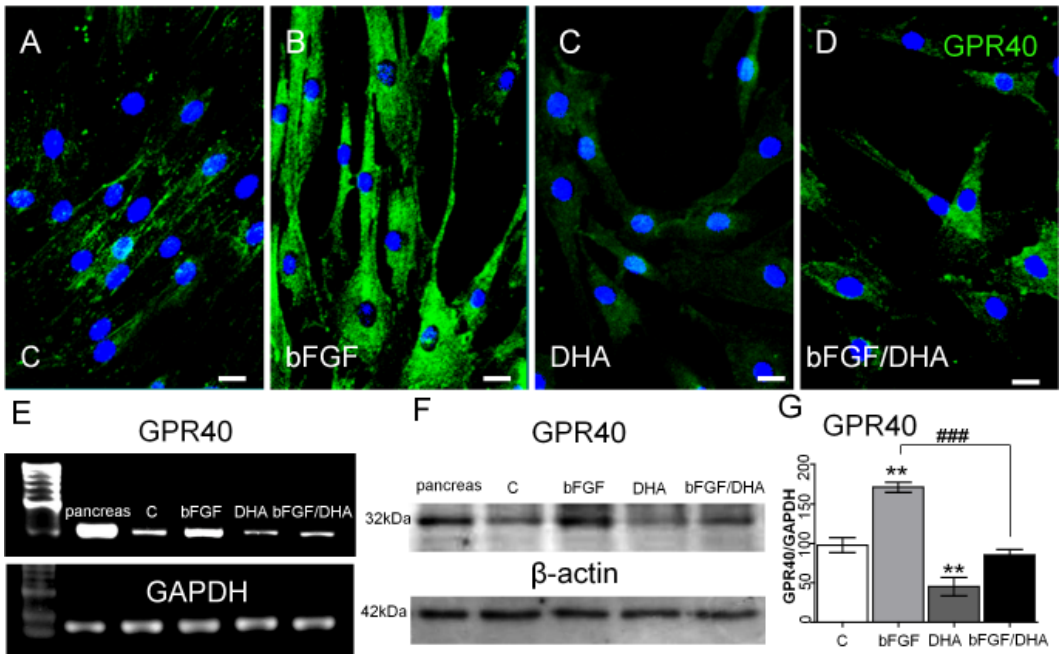

Figure 5. GPR40 expression. Immunofluorescent staining of GPR40 in green. (**A**) Control group, (**B**) bFGF treatment, (**C**) DHA treatment, (**D**) combination bFGF/DHA treatment. Scale bars = 20 μm. (**E**) RT-PCR of GPR40 and (**F**) immunoblots, showed a similar expression pattern. (**G**) Quantifications and ANOVA statistical analysis ($n = 3$, error bars represents S.E.M.) of the RT-PCR results (*) and Bonferroni post hoc test (#) comparing DHA *versus* DHA/bFGF treatments. Values of the GPR40 are calculated as a ratio to GAPDH. **, $p < 0.01$ *vs.* GPR40, control; ###, $p < 0.001$ *vs.* GPR40 - bFGF, GPR40 - bFGF/DHA. Non-induced BMSC had a low expression of the GPR40 gene distributed at the cellular membranes. bFGF treatment led to a significant increase in GPR40 mRNA, and the protein was abundant in the whole cytoplasm and processes. On the contrary, DHA treatment almost completely abolished the GPR40 gene expression. DHA/bFGF treatment showed no changes in expression profile, being compared to the control group. However, a comparison between the groups with bFGF and DHA/bFGF treatments revealed a significant decrease in GPR40 expression in the last group.

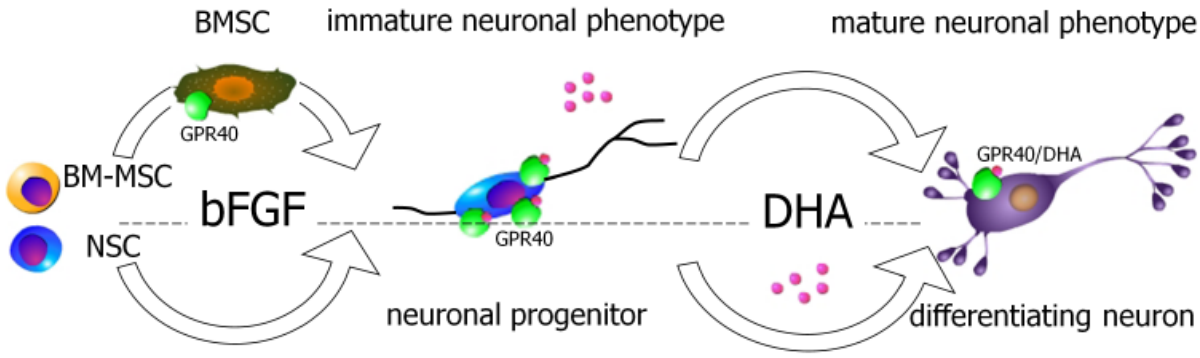

Figure 6. Hypothetical identities between the DHA/GPR40 signaling in BMSC and NSC. Bone marrow-derived stromal cells capable of producing neuronal transcripts and proteins - BMSC (over dashed line) are compared to the neural stem/progenitor cells - NSC (below dashed line). The present data indicate that basic fibroblast grow factor - bFGF may have a role in the phenotype induction in BMSC as found in neuronal stem cells, while DHA could be involved in the subsequent steps of transition to mature phenotype. The latter effects might be linked to the expression of the GPR40 receptor by BMSC.

discovered (*36-38*). After receptor activation, β-arrestins desensitize phosphorylated GPCR, blocking further activation and initiating 'receptor internalization'. GPCR internalization has critical functions to either lysosomal degradative or recycling pathways. Within lysosomes, desensitized GPCR is degraded for the signal termination, whereas in endosomes it is recycled back to the cell surface in a resensitized state (*39,40*). Although responding to diverse agonists, the structural and functional features of GPCR are remarkably conserved. Since protein kinase C (PKC) is involved in the DHA-mediated signal transduction, the 'receptor internalization' leads to its desensitization, without agonist occupancy of the receptor, dependent only on the PKC activation (*39,40*). In our experimental paradigm, GPR40, as a specific receptor for DHA, can probably mediate its signaling and contribute to the enhancement of the bFGF-induced upregulation of mature neuronal marker NF-M and Map2, when DHA was applied together.

It remains uncertain whether BM-MSC, which are known to express bFGF receptor (*21*), can give rise to fully functional neuronal progenitors after bFGF induction *in vitro* (*21,23,41*). However, bFGF alone is capable of inducing a neuronal phenotype from NSC (*42*). As summarized in Figure 6, the present data may point out a potential mechanism of DHA-dependent GPR40 signaling in BMSC and its contribution to the enhancement of bFGF-induced expression of neuronal markers. Additional experiments examining the effect of other PUFA on neural stem cells and neuronal progenitors are needed to clarify the role of PUFA/GPR40 signaling in adult neurogenesis (*10*).

Many features of BMSC remains unclear (*16,18*), but the discovery of their identity will prospectively define their function and elucidate their biological role (*43*). In this regard, DHA signaling through GPR40 may have practical implications by providing a strategy for an efficient pre-treatment of BMSC before their transplantation, since it is now known that BM-MSC expressing neural antigens can instruct a neurogenic cell fate in NSC *in vitro* (*44*) and potentially *in vivo* (*45*). Moreover, it is widely accepted that the transplantation of human MSCs and particularly those capable of producing neuronal transcripts and proteins stimulates proliferation, migration, and differentiation of the endogenous neural stem cells (*46,47*).

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Original Article

Epithelial or mesenchymal: Where to draw the line?

Jianyuan Chai[1,2,*], Cristina Modak[1], Wasim Mouazzen[1], Reinier Narvaez[1], Jennifer Pham[1]

[1] *VA Long Beach Healthcare System, Long Beach, CA, USA;*
[2] *Department of Medicine, University of California, Irvine, CA, USA.*

Summary **Epithelial and mesenchymal cells represent two of the main differentiated cell types in all vertebrates. However, their distinction is not always absolutely clear. Dozens of molecules have been used as markers for each cell type, while emerging evidence questions their validity. The aim of this study was to compare the molecular phenotype of these two cell types. Twenty-two commonly used molecular markers were evaluated by quantitative PCR and immunofluorescence in six lines of human and rat epithelial cells and fibroblasts. The epithelial cells were also examined for their responses to TGFβ1 stimulation. All of the "markers" tested were found in both epithelial and mesenchymal cells. Some epithelial markers, such as CLDN5, OCLN, DSG1 and TJP1, were expressed even higher in fibroblasts than in epithelial cells. In comparison, mesenchymal markers showed more fidelity, but CDH2 and MMP9 were still significantly higher in epithelial cells than in mesenchymal cells. Furthermore, TGFβ1 up-regulated epithelial markers CTNNB1 and CTNND1, but suppressed mesenchymal markers such as S100A4, FGF1 and FGF2. In conclusion, no gene expression is cell-type restricted. Although some of these "markers" are expressed more in one cell type than in the other or differently localized, none of them shows a consistent pattern across species to make them universal markers. Nonetheless, some molecules appear to be better markers than others for a specific cell type. The information provided here is expected to serve as a reference for both basic and clinical researchers in the fields of epithelial-mesenchymal transition, molecular cell typing and cancer diagnosis.**

Keywords: Epithelial cell, mesenchymal cell, EMT, molecular marker

1. Introduction

Epithelial and mesenchymal cells represent two of the main cell types in all vertebrates. Their distinctions in morphology and cellular organization have already been recognized in the late 19th century. Epithelial cells are commonly characterized by their: (*i*) strong intercellular connection; (*ii*) keratin-based cytoskeleton; and (*iii*) distinct cell polarity. In contrast, mesenchymal cells have loose or no intercellular connections among them, contain a highly developed cytoskeletal structure with vimentin as an intermediate filament, and have no clear distinction in either apical, lateral or basal side when they are in rest. However, more and more studies found that these two types of cells are not as rigid as they were thought; and both epithelial and mesenchymal cells show some degree of cell plasticity *in vitro* and *in vivo*, namely, they are interchangeable. This phenomenon is called epithelial-mesenchymal transition (EMT) or in reverse, mesenchymal-epithelial transition (MET).

Although the awareness of EMT and MET phenomena can be dated back as early as 1908 in Lillie's embryological book 'The Development of the Chick' (*1*), their detailed description was not established until 1982 by Greenburg and Hay (*2*). During the 1990s, EMT gained more recognition as a possibly important mechanism in malignant and chronic progression of fibrotic disorders and cancer (*3-7*) and it has been studied extensively ever since. It was during these early years of extensive EMT studies that transforming growth factor beta 1 (TGFβ1) was reported as an inducer of EMT in normal mammary epithelial cells (*8*). Since then, even though other

**Address correspondence to:*
Dr. Jianyuan Chai, Dept Research (09-151), VA Long Beach Healthcare System, 5901 E. Seventh Street, Long Beach, CA 90822, USA.
e-mail: Jianyuan.chai@va.gov

molecules, particularly growth factors and extra cellular matrix molecules, have also been reported to induce or facilitate EMT, TGFβ1 is one of the most recognized inducers of EMT both *in vitro* and *in vivo*, particularly in embryonic development and cancer progression, as well as in epithelial tissue injury (*9*). Through these early benchmark studies, it is now understood that EMT and MET are two essential processes in embryonic development and morphogenesis to facilitate tissue differentiation and organ formation, and these cellular capabilities are kept very well by most vertebrates throughout their adulthood as key mechanisms for wound healing and tissue repair. Although these differentiated cells are not as much versatile as stem cells, epithelial and mesenchymal cells by inter-conversion can compensate for stem cell shortage in tissue repair. Inadvertently, these beautiful features of differentiated cells are sometimes hijacked by "evil forces" when they are deregulated (*3*). As mentioned previously, fibrosis is a typical example in which EMT is overwhelmed and, as a result, normal tissue structure is replaced by excessive mesenchymal cells and their products (*4*). An even worse case, which has been attracting more attention in recent years, is cancer, in which some genetically altered epithelial cells have acquired mesenchymal features to move around in our body, invade our healthy organs and break down their normal functions (*3,5,6*).

In order to prevent unnecessary EMT or MET, it is often required to detect cell type changes as early and precisely as possible. Many molecules have been claimed and used as markers for either epithelial cells or mesenchymal cells. However, an increasing amount of evidence suggests that none of these markers seem to be perfect. This study is to compare the molecular phenotype of epithelial cells with mesenchymal cells in the resting state, to evaluate the molecular markers that are commonly used nowadays to distinguish epithelial cells from mesenchymal cells, and to explore the uniqueness of these two cell types. To provide a positive control for our studies, we also examined these markers in epithelial cells in response to TGFβ1, the most recognized EMT-inducing agent (*9*).

2. Materials and Methods

2.1. *Cell culture*

Three human and three rat cell lines were compared in this study: human esophageal squamous epithelial cells (Het-1A), human gastric adenocarcinoma epithelial cells (AGS), human colon fibroblast (CCD-18Co), rat gastric mucosal epithelial cells (RGM1), rat intestinal epithelial cells (IEC-6), and rat embryonic fibroblast (Rat2). All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) except RGM1 which was obtained from Riken Cell Bank (Tsukuba, Japan). Culture media used for each cell line are: Het-1A – Bronchial Epithelial Growth Medium with bullets and 10% FBS (Lonza, Walkersville, MD, USA), AGS – RPMI 1640 with 10% FBS (Lonza), CCD-18Co – Eagle's Minimum Essential Medium (ATCC) with 10% FBS, RGM1 – Dulbecco's Modified Eagle's Medium/F12 with 10% FBS (Lonza), IEC-6 – Dulbecco's Modified Eagle's Medium (Lonza) supplemented with 0.1 units/mL bovine insulin (Sigma-Aldrich, Saint Louis, MI, USA) and 10% FBS, and Rat2 – Dulbecco's Modified Eagle's Medium with 10% FBS. For all the experiments in this study, comparable cell density was insured by plating equal number of cells and confirming by microscopic visualization prior to serum starvation. All cells were serum starved for 5 h before experiments. Where applicable, after serum starvation, cells were treated with either vehicle (control) or recombinant TGFβ1 (Invitrogen, Camarillo, CA, USA) at 5 ng/mL for 24 h.

2.2. *Real-time PCR*

Cells were cultured in 6-well plates till desired confluence. Total RNA was extracted from the cells using RNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Reverse transcription was done in MyCycler (Bio-Rad, Hercules, CA, USA) following the protocol: 25°C/10 min – 55°C/30 min – 85°C/5 min – 4°C/∞. All of the reagents for reverse transcription, including transcriptor reverse transcriptase, PCR nucleotide mix, protector RNase inhibitor, and random primer p[dN]6, were purchased from Roche (Mannheim, Germany). Real-time PCR was performed in iCycler (Bio-Rad) following two-step program, using SYBR Green master mix and specific primers from SABiosciences (Frederick, MD). C_t readings from epithelial samples were compared against the C_t value from fibroblast within the same species. Data were generated from at least 5 independent experiments and were analyzed according to $\Delta\Delta C_t$ method.

2.3. *Fluorescence microscopy*

Cells were cultured till desired confluence on microscopic cover slips that were pre-coated with 5% rat tail type I collagen (BD Biosciences, San Diego, CA, USA) in 0.02 N acetic acid. Cells were fixed for 10 min in either 4% paraformaldehyde (for membrane-associated antigens) or cold methanol (for cytoplasmic antigens). For cytoplasmic antigens, cells were also permeabilized in cold acetone for 5 min after fixation. Before a primary antibody was applied, cells were incubated in serum-free Protein Block (Dako, Carpentaria, CA, USA) for 30 min to eliminate possible non-specific antibody binding. To determine the specificity of each primary antibody, the primary antibody in control cover slips was replaced

with a pre-immune serum. The primary antibodies used for cell staining include: E-cadherin (1:500), N-cadherin (1:500), desmoglein (1:500), p120 catenin (1:500), and γ-catenin (1:500) all from BD Biosciences; fibronectin (1:200), S100A4 (1:100), vimentin (1:100), cytokeratin-14 (1:200), acidic cytokeratin (1:50), non-muscle myosin heavy chain (1:500), and β-catenin (1:500) all from Abcam (Cambridge, MA, USA); cytokeratin-18 (1:200) from Enzo (Plymouth Meeting, PA, USA); smooth muscle α-actin (1:400) from Sigma-Aldrich; occludin (1:100) and ZO-1 (1:100) from Invitrogen. FITC-conjugated secondary antibodies were purchased from Abcam and diluted in PBS according to the manufacturer's recommendation. Nuclei were counter-stained with either propidium iodide or DAPI (Invitrogen). To show actin polymerization, cells were fixed in 3.7% formaldehyde (Sigma-Aldrich) for 10 min, permeabilized in 0.1% Triton X-100 (Thermo Fisher Scientific, Pittsburg, PA, USA) for 5 min, and incubated with Oregon Green 488 phalloidin (Invitrogen) for 20 min.

3. Results

3.1. *Epithelial markers*

The most prominent feature of epithelial cells is their cohesion and tendency to form continuous cell layers regardless of *in vitro* or *in vivo*. Adjacent epithelial cells are connected to one another through multiple intercellular locks, including tight junctions, adherens junctions, desmosomes and gap junctions, which in turn link to the intracellular cytoskeleton. For this reason, the molecules constituting these membranous structures are commonly used as markers to identify epithelial cells and therefore, they were carefully examined one-by-one in this study.

Tight junctions (TJ) are located in the most apical lateral regions of epithelial cells and thus they are also indicators for cell polarity. The main components of a TJ include transmembrane proteins claudin (23 members known in human), occludin, junctional adhesion molecule (JAM-A, -B, and -C) and intracellular adaptor protein zona occludin (ZO-1, -2, and -3) which connects a TJ to the actin cytoskeleton (*10*).

At the transcriptional level, all TJ components were expressed not only in epithelial cells, but also detectable in mesenchymal cell lines, even though TGFβ1 treatment significantly knocked down expression of most genes in most cell lines (Figures 1 and 2). Nonetheless, claudin-5 (CLDN5), one of the most common claudins in gastrointestinal epithelial cells (*11*), was expressed at much higher levels in human epithelial cells than in human fibroblasts, consistent with what would be expected for a TJ molecule (Figure 1). In rat cell lines, however, it was just the opposite where Rat2 fibroblasts expressed ~2-fold more claudin-5 than the two rat epithelial cell lines (Figure 2). Rat2 fibroblasts also expressed higher levels of occludin (OCLN) than either IEC-6 or RGM1 epithelial cells (Figure 2). Interestingly, in human AGS cells, which expressed a great amount of OCLN, TGFβ1 did not inhibit the gene expression, as it did in all the other cases, but actually promoted it. Finally, the level of TJP1, the gene coding for the first identified TJ protein ZO-1, was found significantly higher in CCD-18Co human fibroblasts than in either Het-1A or AGS human epithelial cells (Figure 1). Immunocytochemical staining confirmed expression of all TJ proteins in both epithelial and mesenchymal cell lines and did not reveal any consistent shift in localization between these two cell types, with the exception of ZO-1 which was exclusively localized to the membrane in all epithelial cells, but showed additional cytoplasmic staining in the two mesenchymal cell lines (Figure 3).

Adherens junctions (AJ) are specialized sub-apical structures that form stable cell-cell contacts in essentially all types of tissue (*12*). Molecules that constitute an AJ include transmembrane protein E-cadherin and intracellular adaptors α-, β-, and δ-catenin (p120), which in turn provide anchorage to the actin cytoskeleton.

Among AJ components, E-cadherin expression is commonly considered a hallmark of epithelial cells. Its expression is mainly controlled at the transcriptional level, seemingly, CDH1, the gene coding for E-cadherin, is normally inactivated in mesenchymal cells (*13*). While this was confirmed for human CCD-18Co fibroblast cells both at the mRNA and protein level (Figures 1 and 3), Rat2 fibroblasts, which appeared to have only small amounts of CDH1 mRNA compared to IEC-6 epithelial cells, still expressed ~10-fold higher levels compared to RGM1 epithelial cells (Figure 2) and stained clearly positive for E-cadherin protein at the membrane (Figure 3). Catenins were not unique to epithelial cells either. Both β-catenin (CTNNB1) and δ-catenin (CTNND1) were expressed at considerable amounts in all cell lines and were even significantly higher in Rat2 fibroblasts than in RGM1 epithelial cells (Figure 2). Interestingly, TGFβ1 treatment promoted CTNNB1 and CTNND1 expression in all epithelial cell lines (Figures 1 and 2).

Furthermore, their proteins were localized predominantly to the cell membrane in all cell lines except the cancerous epithelial cell line – AGS – in which β-catenin was nuclear localized (Figure 3). Rat2 fibroblasts also showed some nuclear staining of β-catenin but still retained strong, defined membrane localization as well (Figure 3). Even though TGFβ1 treatment induced nuclear localization also in human Het-1A and rat RGM1 cells, both rat epithelial cell lines still exhibited strong membrane localization (Supplementary Figure).

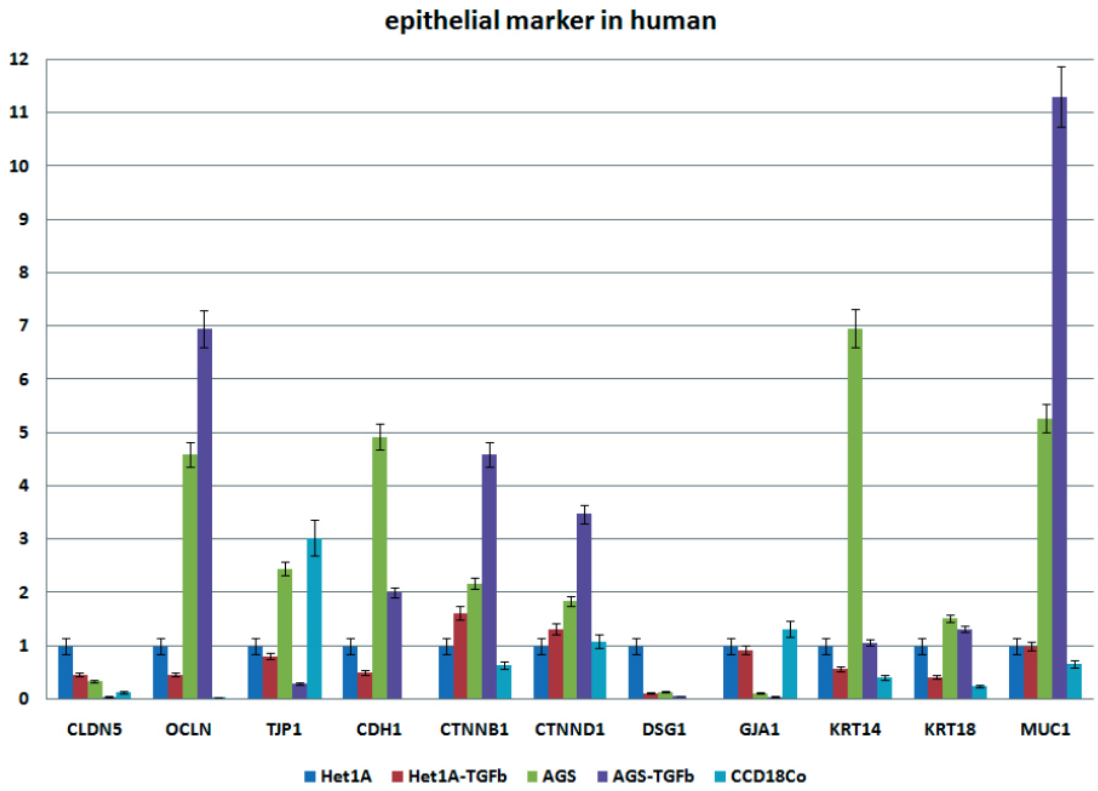

Figure 1. mRNA expression of epithelial markers in human cell lines. Three human cell lines, including esophageal squamous epithelial cells (Het-1A), gastric adenocarcinoma cells (AGS) and colon fibroblasts (CCD-18Co), were examined in their resting state and TGFβ1-induced EMT state. Non-treated Het-1A epithelial cells were set as a standard in the graph and the rest of the cells were compared against it. Results represent averages from at least three independent experiments with error bars representing standard error. $p < 0.01$.

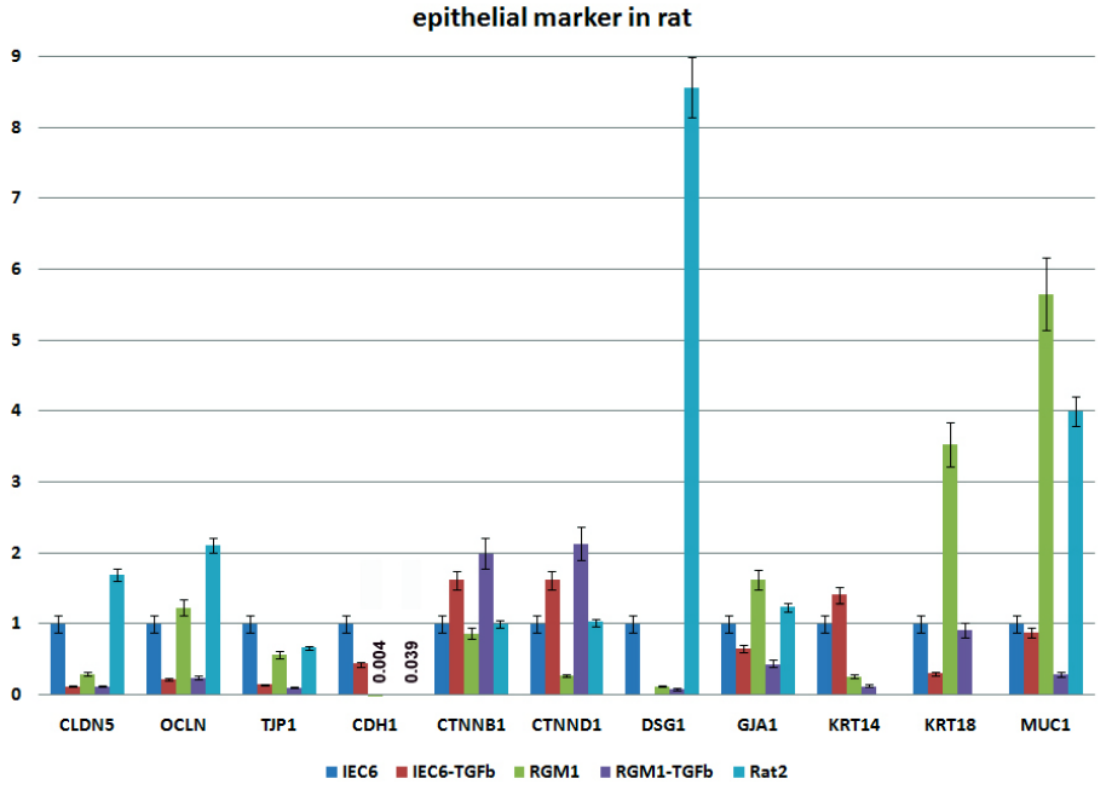

Figure 2. mRNA expression of epithelial markers in rat cell lines. Three rat cells lines, including intestinal columnar epithelial cells (IEC-6), gastric mucosal columnar epithelial cells (RGM1) and embryonic fibroblasts (Rat2), were examined in their resting state and TGFβ1-induced EMT state. Non-treated IEC-6 epithelial cells were set as a standard in the graph and the rest of the cells were compared against IEC-6. Results represent averages from at least three independent experiments with error bars representing standard error. $p < 0.01$.

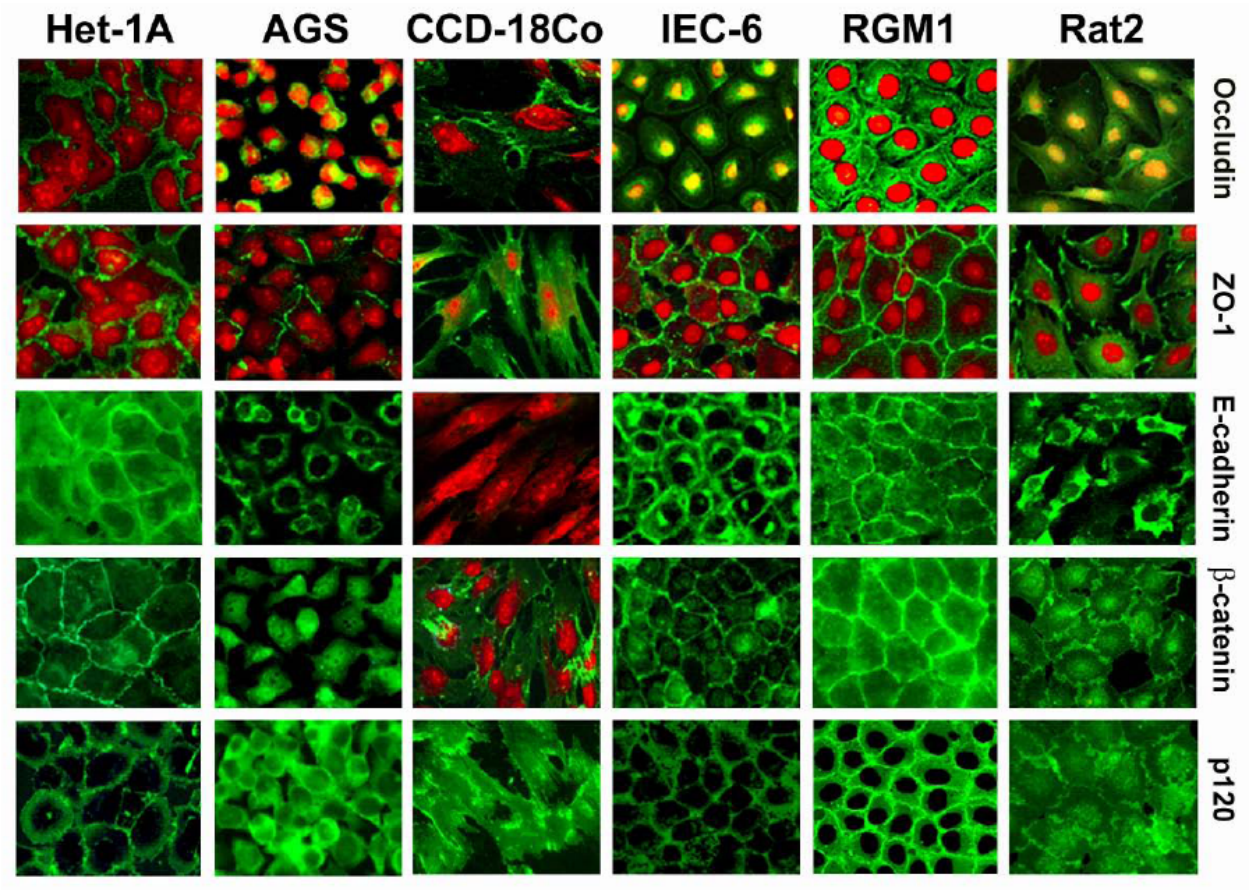

Figure 3. Immunofluorescence detection of protein localization of epithelial markers in human (Het-1A, AGS, CCD-18Co) and rat cell lines (IEC-6, RGM1, Rat2). Localization patterns of putative epithelial markers Occludin, ZO-1, E-cadherin, β-catenin and p120 were evaluated as described in the methods section. Nuclei were counterstained with propidium iodide (red) as needed.

Desmosomes are like buttons joining the lateral edges of adjacent epithelial cells through cadherin molecules desmoglein and desmocollin which link with cytokeratin fibers through desmosomal plaque proteins such as desmoplakin and γ-catenin (Plakoglobin).

Although desmoglein (DSG1) was not expressed in human CCD-18Co fibroblasts (Figure 1), its expression in Rat2 fibroblasts was found extremely higher than in either rat epithelial cell line (Figure 2). Immunofluorescence cell staining showed strong membrane localization of γ-catenin in both epithelial and mesenchymal cell lines (Figure 4). Similar results were obtained for desmoglein, with no clear distinction in localization patterns between epithelial and mesenchymal cells. On the other hand, TGFβ1 had a suppressive effect on desmosomes components (Figures 1 and 2).

Gap junctions are channel-like structures each composed of two connexons which connect across the intercellular space and regulate trafficking of small molecules (< 1 kDa) between adjacent cells. Each connexon is a pore through the cell membrane and is formed by a ring of six connexin proteins (> 20 isoforms identified). Connexins are expressed by virtually all types of cells, including fibroblasts, except sperms and erythrocytes (*14-17*).

Our data showed expression of connexin-43 (GJA1) in all six cell lines examined. Moreover, its mRNA level in CCD-18Co fibroblasts was significantly higher than in either Het-1A or AGS epithelial cells (Figure 1). It was also slightly higher in Rat2 fibroblasts than in IEC-6 epithelial cells (Figure 2). TGFβ1 had an inhibitory effect on GJA1.

Keratins are intermediate filament proteins found in the epithelial tissue. There are at least 20 different keratin isoforms in two groups found together in pairs which characterize the type of epithelium: the low weight, acidic type I keratins and the high weight, basic or neutral type II. Thus this specific keratin fingerprint is commonly used to classify all epithelia upon their cytokeratin expression profile.

In our study, no keratins were found in Rat2 fibroblasts, but CCD-18Co fibroblasts expressed both keratin-14 (KRT14) and -18 (KRT18) at the transcriptional level, even though they were overall relatively low (Figure 1). On the other hand, cell staining for acidic keratins (A-keratin) showed positive only in epithelial cells (Figure 4). In addition, consistent with keratins being epithelial markers, TGFβ1 treatment significantly reduced expression of both keratins in

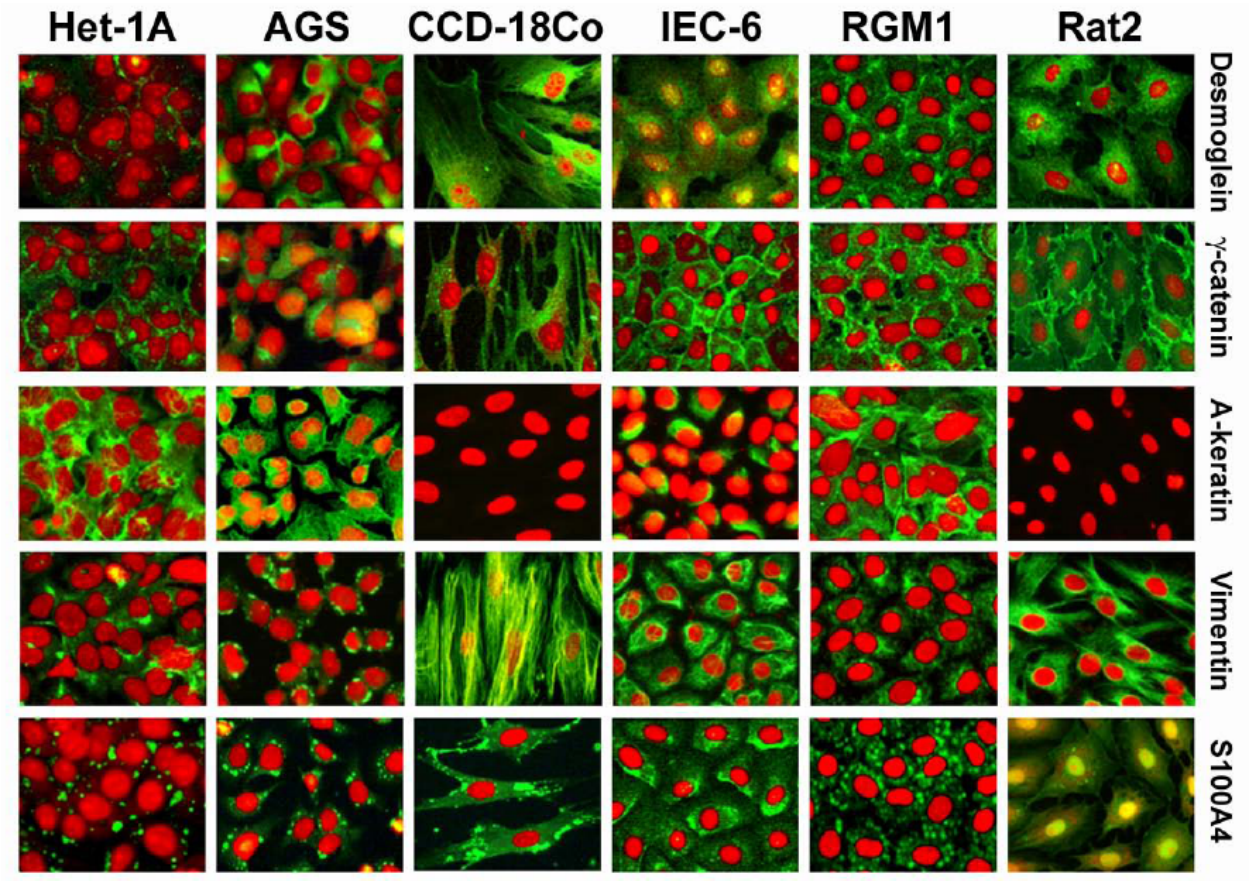

Figure 4. Immunofluorescence detection of protein localization of epithelial and mesenchymal markers in human (Het-1A, AGS, CCD-18Co) and rat cell lines (IEC-6, RGM1, Rat2). Localization patterns of putative epithelial markers (desmoglein, γ-catenin, and acidic keratin) and mesenchymal markers (vimentin and S100A4) were evaluated as described in the methods section. Nuclei were counterstained with propidium iodide (red) as needed.

most epithelial cell lines, with the exception of KRT14 in IEC6 cells (Figures 1 and 2).

Mucins are glycoproteins produced and mostly secreted by epithelial cells to protect themselves against pathogens from outside. At least 19 isoforms have been found in human, some of which are transmembrane proteins, for example, mucin-1. Mucin-1 is localized to the apical side of the epithelium and its cytoplasmic domain binds to the actin cytoskeleton, and therefore mucin-1 is also used as an epithelial polarity marker.

In our study, mucin-1 (MUC1) expression was detectable in all six cell lines examined, with no consistent trend difference between epithelial and mesenchymal cells (Figures 1 and 2). For instance, in Rat2 fibroblasts MUC1 was expressed significantly higher than in IEC-6 epithelial cells but lower than in RGM1 epithelial cells. Moreover, its expression in AGS cells was significantly increased in response to TGFβ1.

3.2. *Mesenchymal markers*

Mesenchymal cells are known for having advanced cytoskeletal structure, high motility, and for producing more extracellular matrix proteins compared to epithelial cells.

Vimentin is an intermediate filament protein that has been widely used as a molecular marker for mesenchymal cells. However, numerous data have shown that vimentin can also be expressed in epithelial cells involved in physiological processes requiring epithelial cell migration, such as placentation and wound healing.

In our study, vimentin (VIM) mRNA expression was found in all the cell lines except AGS, although it was significantly higher in both human and rat fibroblasts than in epithelial cells within the same species (Figures 5 and 6). Its protein expression was also detected in all the epithelial cells including AGS cells (Figure 4), but it did not show typical filamentous phenotype in either Het-1A or AGS as seen in fibroblasts. On the other hand, all rat cell lines expressed high levels of vimentin protein independent of cell type (Figure 4). TGFβ1 increased VIM expression in all epithelial cells (Figures 5 and 6), which displayed a more typical filamentous phenotype as well (Supplementary Figure).

S100A4, also known as fibroblast-specific protein (FSP1), was once described as an absolute marker for fibroblasts (*18*). Any cells that express S100A4 protein and/or show S100A4 promoter activity were classified as fibroblasts. However, more and more evidence

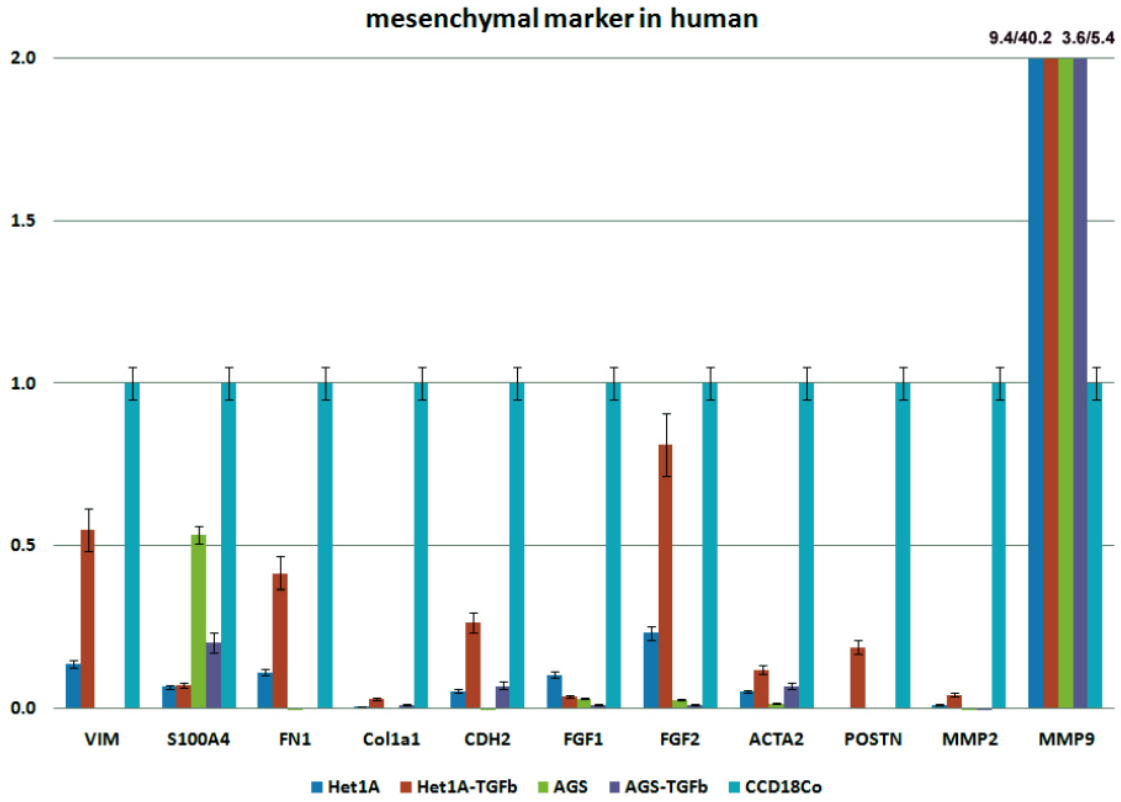

Figure 5. mRNA expression of mesenchymal markers in human cell lines. Three human cell lines (Het-1A, AGS and CCD-18Co) were examined in their resting state and TGFβ1-induced EMT state. Non-treated CCD-18Co fibroblasts were set as a standard in the graph and the rest of the cells were compared against it. Results represent averages from at least three independent experiments with error bars representing standard error. $p < 0.01$.

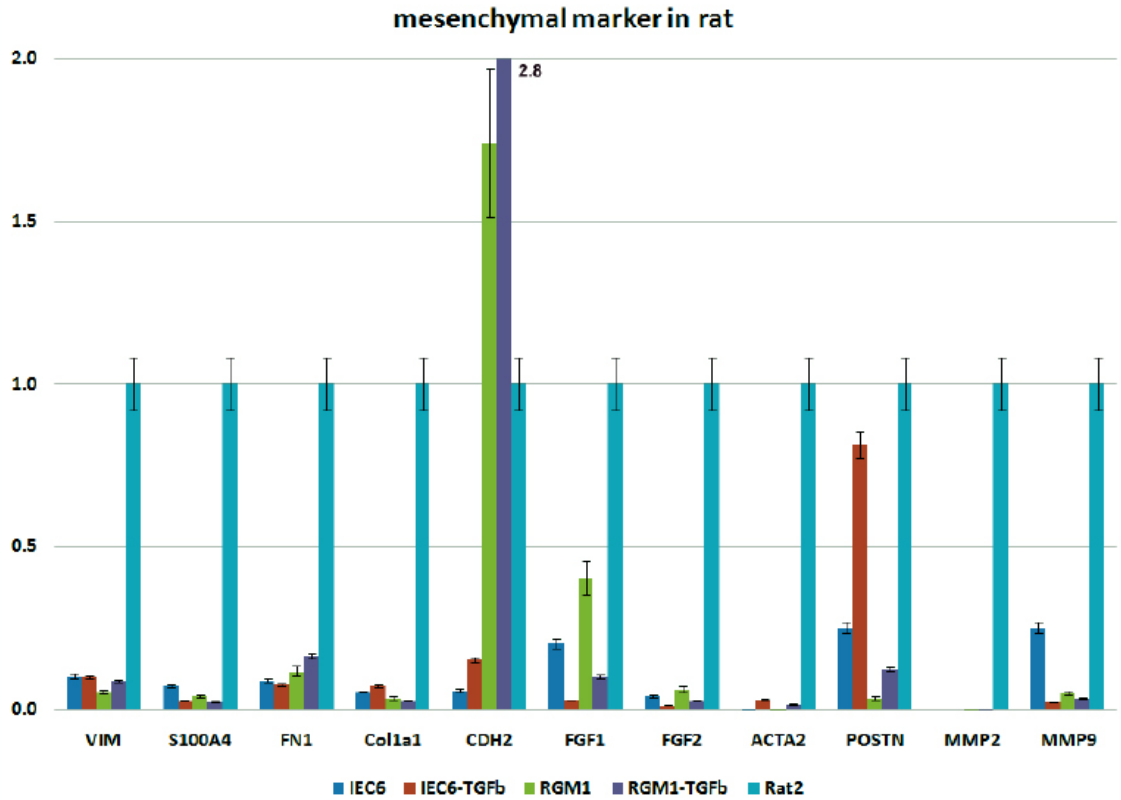

Figure 6. mRNA expression of mesenchymal markers in rat cell lines. Three rat cells lines (IEC-6, RGM1 and Rat2) were examined in their resting state and TGFβ1-induced EMT state. Non-treated Rat2 fibroblasts were set as a standard in the graph and the rest of the cells were compared against it. Results represent averages from at least three independent experiments with error bars representing standard error. $p < 0.01$.

shows that S100A4 is also expressed in other cell types, including blood cells, platelets, endothelial cells, smooth muscle cells, cardiomyocytes, astrocytes, and biliary epithelial cells (*19*).

In our study, S100A4 mRNA expression was found in all the cell lines that were studied, even though expression levels were relatively low for most epithelial cell lines, with the exception of AGS cancer cells, which expressed considerable amounts, albeit still lower than in fibroblasts (Figures 5 and 6). Surprisingly, TGFβ1 strongly inhibited S100A4 expression in 3 out of 4 epithelial cell lines, with the exception of Het-1A cells, raising more questions about the eligibility of S100A4 as a mesenchymal marker. S100A4 protein expression was also clearly detected in all the six cell lines, with rat epithelial RGM1 cells expressing comparable amounts to fibroblasts (Figure 4).

Fibronectin exists in 2 forms, plasma fibronectin and cellular fibronectin. Plasma fibronectin is synthesized by hepatocytes and represents about 1% of serum protein, while cellular fibronectin is made by many different cell types, including fibroblasts, chondrocytes, endothelial cells, macrophages, as well as certain epithelial cells (*20*). The protein is deposited in the extracellular matrix as highly insoluble fibronectin filaments.

In our study, cellular fibronectin (FN1) was expressed in all the cell lines examined, although it was significantly higher in mesenchymal cells than in epithelial cells (Figures 5 and 6). Cell staining confirmed that fibronectin protein was produced and secreted by five out of six cell lines with no clear distinction in expression pattern (Figure 7). Its expression in AGS cells was not detectable, and TGFβ1 treatment, which promoted FN1 expression in most other epithelial cell lines, did not induce it either.

Collagens are the main component of connective tissue and also the main protein of the extracellular matrix that supports other tissues. They are primarily synthesized by fibroblasts. Among 29 types of collagens known so far, type I collagen is the most abundant one in the human body.

In this study, we examined expression of COL1A1 – the gene coding for the α-subunit of type I collagen. Although all six cell lines expressed COL1A1, it was drastically higher in mesenchymal cells than in any epithelial cells (Figures 5 and 6). TGFβ1 treatment modestly promoted expression in most epithelial cell

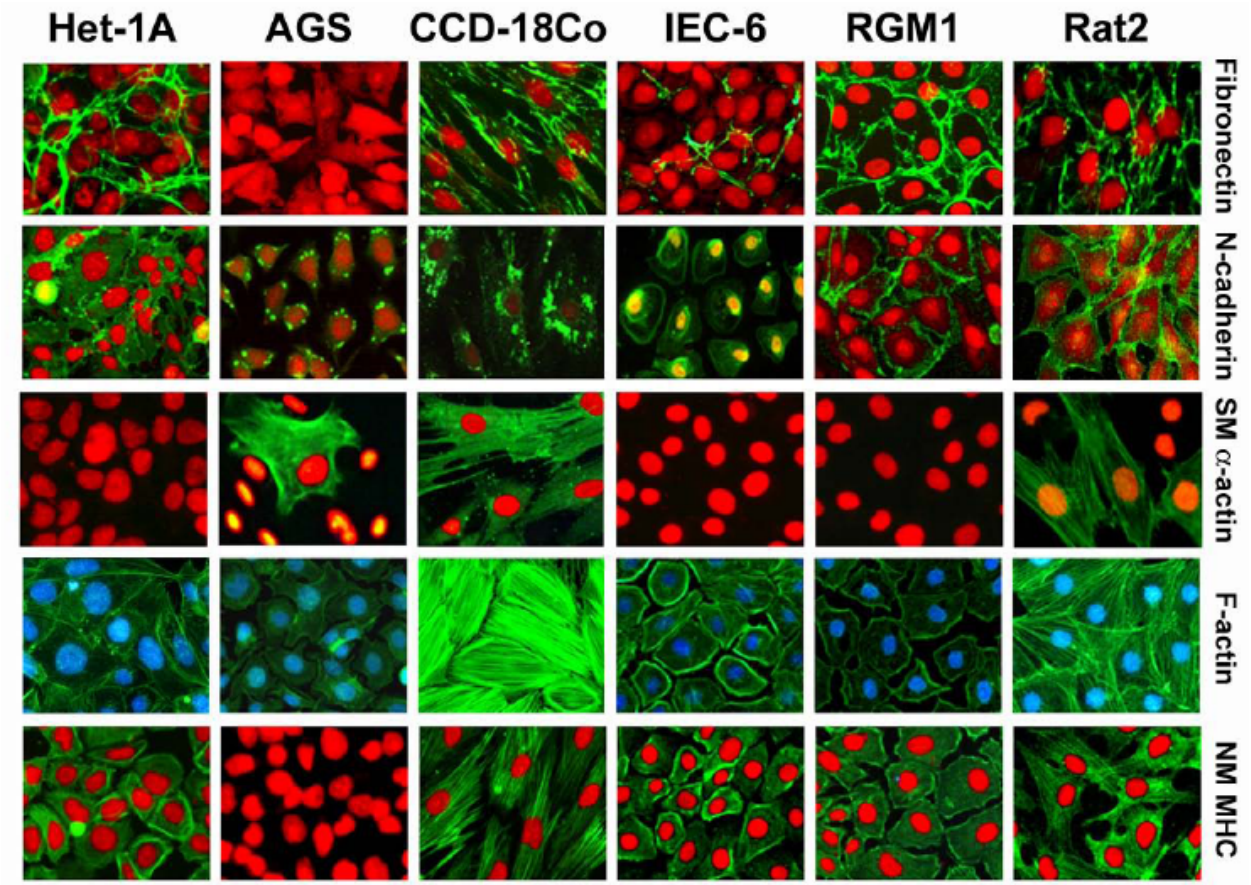

Figure 7. Immunofluorescence detection of protein localization of mesenchymal markers in human (Het-1A, AGS, CCD-18Co) and rat cell lines (IEC-6, RGM1, Rat2). Localization patterns of putative mesenchymal markers (fibronectin, N-cadherin and SM α-actin) were evaluated as described in the methods section. Nuclei were counterstained with either propidium iodide (red) or DAPI (blue) as needed. F-actin and non-muscle myosin heavy chain (NM MHC) were stained to confirm that all the non-treated epithelial cells were not undergoing EMT.

lines (Figures 5 and 6).

N-cadherin is normally found in neural tissue, retina, endothelial cells, fibroblasts, osteoblasts, mesothelium, myocytes, limb cartilage, oocytes, spermatids and Sertoli cells. Switch from E-cadherin to N-cadherin expression in epithelial cells is often considered a sign of EMT (*21*). While this switch is an integral part of several processes during normal development, aberrant expression of N-cadherin by cancer cells contributes to their invasiveness and metastasis in various tissues, including breast, thyroid, bladder, prostate and pancreas (*22,23*), making this a widely used marker for EMT and cancer studies.

In our study, mRNA expression of N-cadherin (CDH2) was found in all the cells examined except AGS, even though its level in Het-1A cells was also very low compared to its expression in CCD-18Co fibroblasts (Figure 5). On the contrary, CDH2 expression in RGM1 epithelial cells was found significantly higher than in Rat2 fibroblasts (Figure 6). TGFβ1 promoted CDH2 expressions in all the epithelial cell lines. Cell staining showed positive membrane localization in all six cell lines, including AGS (Figure 7).

Fibroblast growth factors (FGFs) are a group of over 25 small mitogenic proteins for fibroblasts. Acidic FGF (FGF1) and basic FGF (FGF2) are the prototypes of the FGF family.

In our study, both FGF1 and FGF2 were identified in all cell lines examined; moreover, TGFβ1 had an inhibitory effect on FGF1 in all the epithelial cells and also knocked down FGF2 in three out of four epithelial cell lines (Figures 5 and 6).

Smooth muscle α-actin (SM α-actin) is traditionally used as a marker to distinguish myofibroblasts from regular fibroblasts (*21*).

At the transcriptional level, our data showed that SM α-actin (ACTA2) was expressed in every cell line examined (Figures 5 and 6), although it was significantly higher in mesenchymal cells than in any epithelial cell lines. Cell staining showed that 40-60% of CCD-18Co and Rat2 cells were positive for SM α-actin. In epithelial cells, while there was still detectable signal, it was weak and not in filamentous phenotype as seen in mesenchymal cells (Figure 7). TGFβ1 increased ACTA2 expressions in all the epithelial cells.

Periostin (POSTN), also known as osteoblast-specific factor 2, is a secreted, homodimeric protein synthesized by mesenchymal cells such as smooth muscle cells, fibroblasts and osteoblasts, as well as in the periosteum and periodontal ligament. Recent clinical evidence has revealed that periostin is involved in the development of various tumors, such as breast, lung, colon, pancreatic, and ovarian cancers where it promotes EMT, invasion, and metastasis (*25*).

In our study, POSTN was not detectable in AGS cells, but it was expressed in all the remaining epithelial cells (Figures 5 and 6). Its expression was greatly up-regulated by TGFβ1 in all the epithelial cells.

Matrix metalloproteinases (MMPs) are a group of over 25 proteases, either secreted or membrane-associated, that rely on metal ions for their catalytic activity. Collectively MMPs are capable of degrading all kinds of extracellular matrix components, therefore, their expression level is critical to embryonic development, tissue remodeling and cancer metastasis. Mesenchymal cells are the main source of MMPs and, for this reason, expression of MMPs in epithelial cells is often considered an indicator of EMT (*26,27*).

Most MMPs are tightly regulated at the transcriptional level. In our study, we measured expression of MMP2 (gelatinase A) and MMP9 (gelatinase B), which are closely correlated with EMT and cancer metastasis (*28*). MMP2 expression was undetectable in IEC-6 cells and was still minimal in the rest of the epithelial cell lines compared to its level in the fibroblasts of the same species (Figures 5 and 6). The expression of MMP9, however, was significantly higher in both human epithelial cell lines than in CCD-18Co fibroblasts (Figure 5). TGFβ1 had a positive effect on both MMPs.

When epithelial cells are in the process of EMT, they do not only express mesenchymal markers, but also increase their mobility, which can be reflected by formation of actin and myosin stress fibers. To confirm that the epithelial cell lines used in this study were not undergoing EMT when examined in their rest phase, which could have otherwise explained improper expression of some putative mesenchymal markers, we stained the cells for filamentous actin (F-actin) and nonmuscle myosin heavy chain (NM-MHC). In both CCD-18Co and Rat2 fibroblasts, extensive actin and myosin stress fibers were found. In contrast, actin and myosin in the epithelial cells only displayed cortical distribution (Figure 7), while AGS cells did not express NM-MHC at all. In contrast, when the same epithelial cell lines were subjected to TGFβ1 treatment to induce EMT, they all displayed strong actin and, to a lesser extent, myosin stress fibers (Supplementary Figure), suggesting that all the epithelial cells used in this study were not undergoing EMT when the experiments were conducted.

4. Discussion

Dozens of molecules have been commonly used by both basic scientists and clinical researchers as markers to distinguish epithelial and mesenchymal cells for different applications, including molecular cell typing and cancer diagnosis. However, emerging evidence questions the validity of these molecular identifiers. Among epithelial markers, Claudin-5 has been identified in cardiomyocytes (*29*); JAM-A and JAM-C have been reported in fibroblasts derived from various tissues including derma, lung, cornea, and embryo

(*30*); ZO-1 is not only associated with cell membrane of corneal fibroblasts, but it can also translocate to the nucleus to serve as a transcription cofactor in case of corneal injury (*31*); Mucin-1 is also expressed in myofibroblasts (*32*); and all four catenins (α, β, γ, and δ) have been documented in fibroblasts (*33,34*). In addition, all of the desmosome proteins are expressed in periodontal ligament fibroblasts (*35*), dental pulp fibroblasts (*36*) and other non-epithelial cells. For example, desmoplakin interacts with cytokeratin filaments in epithelial cells, but also binds to desmin in cardiomyocytes and to vimentin in fibroblasts. E-cadherin expression is commonly considered as a gold seal of epithelial cells. Modulation of E-cadherin expression levels has been vastly used as a key theme of epithelial plasticity and cancer metastasis. However, E-cadherin expression is still maintained in most differentiated tumors, including carcinomas of the skin, head and neck, esophagus, breast, lung, liver, colon, and prostate. Furthermore, our study has shown that E-cadherin is not only expressed in rat embryonic fibroblasts, but its level in those cells is consistently higher compared to rat gastric epithelial cells. Similarly, keratin subtype expression patterns are commonly used as markers to identify different types of epithelial malignancies, as the keratin profile tends to remain constant when an epithelium undergoes malignant transformation. However, keratins have been also found in non-epithelial cells (*37*). In our study, KRT-14 transcription was found in human colon fibroblasts, even though its expression level was significantly lower than that of both human epithelial lines examined.

For mesenchymal cells, as Hay once said (*38*), "There are, in fact, no specific biochemical markers by which we can define the mesenchyme". Among the mesenchymal markers that we tested, vimentin is regularly expressed in ocular epithelial cells together along with cytokeratin (*39,40*); S100A4 is reported in biliary epithelial cells (*41*); Periostin has been detected at high expression levels in both endometrial (*42*) and mammary epithelial cells (*43*); and fibronectin is not only made by fibroblasts, but also produced by many other cell types including certain epithelial cells (*20*). N-Cadherin is another commonly used mesenchymal marker in EMT studies and the switch in expression from E-cadherin to N-cadherin is considered a hallmark of EMT progression and, to some extent, cancer cell invasion (*21,23*). However, several types of epithelial cells express N-cadherin naturally and the E/N switch in cancer cell invasion is not always followed. For example, N-cadherin is required to maintain corneal limbal epithelial progenitor cells (*44*). Moreover, ovarian surface epithelial cells normally express N-cadherin, but switch to E-cadherin during progression to the neoplastic state (*45*), suggesting that an N/E switch rather than E/N switch might play a role in the initiation of ovarian carcinogenesis (*23*). On the other hand, N-cadherin and E-cadherin are concurrently expressed in the epithelial cells of intra-hepatic bile ducts in normal liver (*46*).

In our current study we provide a detailed analysis of all of these putative epithelial and mesenchymal markers and show that none of them exhibit exclusive epithelial or mesenchymal expression, supporting the emerging evidence that none of the molecular markers commonly used to distinguish epithelial cells from mesenchymal cells is universally unique to one cell type. They are all detectable in both cell types and, while some differences in expression and/or localization were observed, these were also not consistent enough throughout the cell lines studied to classify them as universal markers. These two cell types represent two poles and there are numerous intermediate states or subtypes of cells in between. Although they are differentiated cells, they are also interchangeable depending on environmental condition. Change from epithelial state to mesenchymal state or vice versa is a dynamic process. It is a cumulative effect of different expression levels of multiple genes that ultimately sets these two phenotypes apart.

Moreover, despite the great similarity between human and rat, our results clearly indicate that there may still be some cross-species differences that make our quest for universal markers even harder. On the other hand, taking into account that the line between epithelial and mesenchymal identity is not as rigid as it is usually thought to be, we can still identify some molecules that better serve the purpose. Based on our data, keratins appear to be the most consistent epithelial marker among the different candidates tested. Both keratin-14 and -18 are absolutely omitted in Rat2 fibroblasts and are expressed at much higher levels in human epithelial cells compared to corresponding mesenchymal cells CCD-18Co, which express only moderate amounts. Consistent with their epithelial phenotype, TGFβ1 treatment, which promotes EMT, drastically reduced expression of both keratins in most epithelial cell lines. Moreover, even though keratins can be detected at the transcriptional level in CCD-18Co fibroblasts, their protein products are not found, as seen from A-keratin staining, which is exclusive to the epithelial cells in both human and rat. E-cadherin would be our next choice, due to some differences across species. E-cadherin exhibits strong epithelial preference in the human panel; it is expressed in significant amounts in both epithelial cell lines and clearly localized to the membrane but it is absolutely not expressed in human CCD-18Co cells, making it also a good epithelial marker for human cells. Its expression in Rat2 fibroblasts, on the other hand, was found to be higher than in RGM1 epithelial cells, but one should acknowledge the fact that in both these cell lines CDH1 levels are extremely low. Again, the EMT inducer TGFβ1 had a negative effect on CDH1 expression

in all cells examined, further supporting the role of E-cadherin as an epithelial marker. Finally, even though ZO-1 (TJP1) was expressed at high levels in human fibroblasts, it was mainly localized to the cytoplasm, unlike in epithelial cells in which it was exclusively localized to the membrane. So was true with rat cells. TGFβ1 treatment also led to a loss of membrane localization in most epithelial cells, supporting the notion that membrane-to-cytoplasm localization of ZO-1 could also be an informative marker for EMT.

Compared to epithelial markers, mesenchymal markers showed higher fidelity. The majority of mesenchymal markers, except CDH2 and MMP9, exhibited a clear mesenchymal preference with little expression in epithelial cells. Moreover, even though MMP9 was expressed significantly higher in both human epithelial cell lines than in human fibroblasts and RGM1 cells expressed higher levels of CDH2 compared to Rat2 cells, both of these genes were up-regulated by TGFβ1 treatment, consistent with a mesenchymal phenotype. Among all the mesenchymal markers, MMP2 exhibits the strongest distinction, with only very little expression in the epithelial lines examined. COL1A1 is next, with a similar, if not as striking, trend across both species. POSTN and FGF1 also showed very good trends within the human panel. However, while POSTN could still be considered a viable marker in rat cells, FGF2 proved a much better marker in rat cells than FGF1. Moreover, while TGFβ1 treatment promoted POSTN expression in most epithelial cell lines, it actually had a mostly negative effect on both FGF1 and FGF2, raising additional questions about the validity of either FGF as a mesenchymal marker. Vimentin also has significantly higher mRNA expression in fibroblast cells compared to epithelial cells across species but the difference in staining pattern is much more dramatic in the human panel than in the rat panel. On the other hand, although ACTA2 expression in mesenchymal cells is also found overwhelmingly higher than in epithelial cells, it might be due to myofibroblast differentiation.

In summary, despite the limited number of cell lines (mostly of gastrointestinal origin) used in this study, our data support the conclusion that there is no universal molecular marker that can be absolutely unique to either epithelial cells or mesenchymal cells, especially in the light of some differences across species. Nonetheless, among the various markers analyzed, keratins appear to be the No.1 choice for epithelial cells, while E-cadherin is next, depending on the species studied. Among the mesenchymal markers, most, but not all, of them are predominantly expressed by mesenchymal cells, providing a better range of choices. MMP2 would be the preferred marker, COL1A1 would be next. Vimentin and POSTN could also be good alternative markers, while FGFs appear to have clear species differences, where FGF1 is better as a human marker and FGF2 as a rat marker, and could not be recommended as first/sole choice. Loss of ZO-1 membrane localization and/or cytoplasmic redistribution could also be used as a mesenchymal marker, even though it would also not be recommended as a first/sole choice given other alternatives. These recommendations should however be used with caution, taking into account that they were based on data collected from immortalized cell lines. Variations may be found *in vivo* or in primary cell systems. Researchers are advised to take extra precaution when choosing molecular markers to define a cell type.

Finally, multiple molecules involved in EMT can be envisioned as targets of anti-EMT therapy to prevent or restrain invasion and metastasis of cancer cells. Given the complexity of the molecular and cellular pathways leading to EMT, a forced stimulation of MET can be a very neat approach to control EMT. In order to achieve this, however, it is imperative for us to gain a deeper understanding of this dynamic process through a detailed characterization of its different steps and components. Our study here provides ample data in that direction.

Acknowledgements

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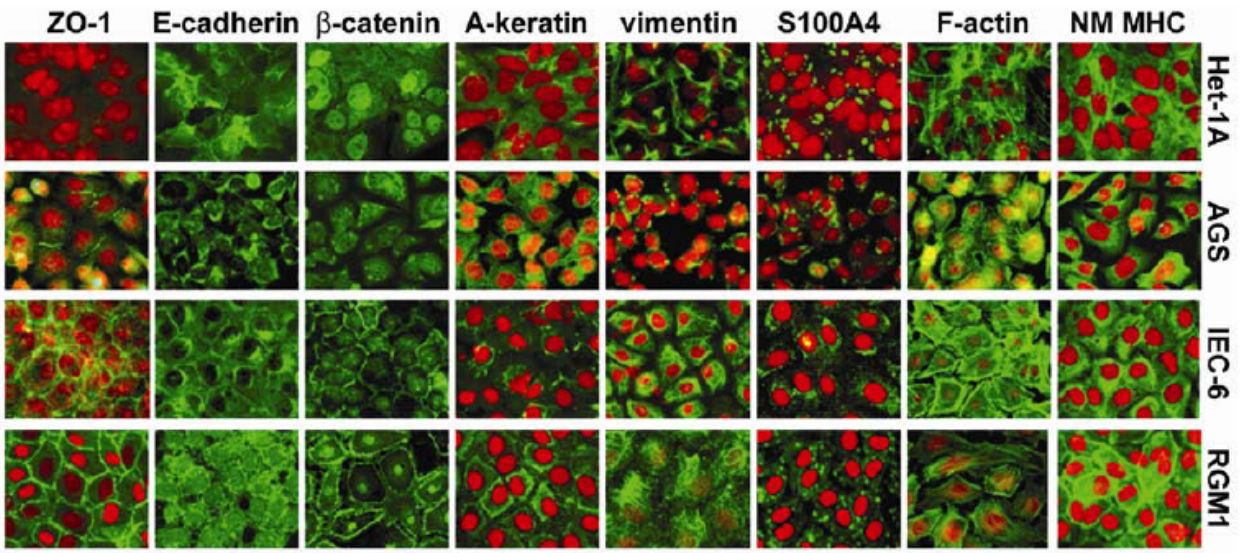
Appendix



Supplementary Figure. Immunofluorescence detection of protein localization of epithelial and mesenchymal markers in TGFβ1-treated human and rat epithelial cell lines. Human (Het-1A, AGS) and rat (IEC-6, RGM1) epithelial cell lines were treated with TGFβ1 to induce EMT. Localization patterns of putative epithelial (ZO-1, E-cadherin, β-catenin and acidic keratin) and mesenchymal markers (Vimentin and S100A4) were evaluated by cell staining as described in the methods section. Nuclei were counterstained with either Propidium Iodide (red) or DAPI (blue) as needed. F-actin and NM MHC fiber-like staining was used as a positive control for EMT.

Case Report

Carcinoid crisis 24 hours after bland embolization: A case report

Sayuri Fujie, Wendy Zhou, Patricia Fann, Yun Yen*

Department of Molecular Pharmacology, City of Hope National Medical Center, Duarte, CA, USA.

Summary **Within 24 h of bland embolization of carcinoid liver metastasis, patient developed flushing and severe hypotension consistent with carcinoid crisis. Octreotide pre- and post-procedure remains the mainstay for prevention and treatment of carcinoid crisis.**

Keywords: Carcinoid syndrome, carcinoid crisis, octreotide, prevention

1. Introduction

Carcinoid crisis is caused by massive acute release of neuroendocrine substances, and patients with carcinoid syndrome are at risk for developing a carcinoid crisis during surgery or other types of intervention. The patients may have sudden change of blood pressure, bronchospasm, facial flushing and even death. Our patient presented with carcinoid crisis 24 h after bland embolization of liver metastases. Pre-procedure administration of octreotide remains the mainstay of preventive therapy for patients undergoing procedures relating to carcinoid tumors. For patients with extensive metastases, adjunct therapy with corticosteroids, cyproheptadine, and glucagon should be considered.

2. Case report

A 75-year-old male was initially found to have multiple liver lesions identified on chest X-ray at time of stress test and echocardiogram in 2005. Ultrasound-guided fine needle aspiration performed in January 2005 showed metastatic carcinoid tumor. He underwent a right hemicolectomy in September 2005, followed by subsequent chemoembolization of his liver metastases. In September 2008, an octreotide scan was done which showed probable progression of his metastatic hepatic disease. Subsequently, the patient underwent another chemoembolization in October 28, 2008. He had intermittent problems with anasarca and continued octreotide therapy until September 4, 2009. As his disease progressed, severe edema, respiratory distress due to pleural effusion, and renal compromise as shown by increasing creatinine and protein spillage further complicated his condition. Bland embolization was performed on January 20, 2010, which involved embolization of the main right hepatic lobe. The following day, the patient developed hypotension 48/30 mmHg and erythema of his face. He was subsequently transferred to the ICU. Patient was given octreotide drip 50 mcg/h, lasix 20 mg/h, dopamine, and levophed drip. Morphine was added for pain control and amiodarone was given for his atrial dysrhythmia. Dialysis was initiated for his renal failure. Patient subsequently developed episodes of severe hypotension, altered mental status with delirium. Beta-blockers, vasopressin, and cyproheptadine were added to therapy and octreotide was increased to 150 mcg/h. Morphine was removed to avoid carcinoid crisis. Patient was transferred to hospice for end-of-life care after developing right hydropneumothorax with complete collapse of the right lung.

3. Discussion

Carcinoid tumor arises from the diffuse neuroendocrine cells of the intestinal tract with the unique ability to secrete bioactive amines and peptides. Prevalence of carcinoid tumor is estimated to be two cases per 100,000 persons in the United States (*1*). Majority of the tumor are found incidentally during other surgical procedures. Carcinoid tumors can cause carcinoid syndrome which is caused by chronic release of neuroendocrine substances such as serotonin, histamine, kallikreins, and catecholamines produced by the carcinoid tumor. The syndrome can be divided into 2 categories: typical and atypical. Typical carcinoid syndrome (TCS) is usually caused by metastatic midgut

**Address correspondence to:*
Dr. Yun Yen, Beckman Research Institute, City of Hope Comprehensive Cancer Center, Beckman Center, Room 4117, 1500 East Duarte Road, Duarte, CA 91019, USA.
e-mail: yyen@coh.org

carcinoids. Atypical carcinoid syndrome (ACS) is most often associated with metastatic foregut carcinoids. Both TCS and ACS cause flushing but the pattern is patchier in ACS. Additionally, these areas of flushing often exhibit central clearing and increased pruritus when compared with flushing due to TCS. Due to the increased systemic levels of histamine often present in foregut carcinoids, bronchoconstriction, salivary gland swelling, cutaneous edema, and lacrimation may also be present (*2*). Only 10% of all patients with carcinoid tumor develop carcinoid syndrome (*3*).

Pre-, peri-, and post-operative care for carcinoid tumor varies. Some advocate octreotide for patients who have carcinoid syndrome and are planning for surgical removal (*4*), while others recommend octreotide for all patients with carcinoid tumors who are planning for surgery (*5*). In addition, H1 receptor blockers, H2 blockers, and occasionally, dexamethasone may be needed if symptoms are severe. It is recommended that patients be evaluated with echocardiogram prior to surgery for the presence of carcinoid heart disease. Electrolytes should be obtained in patients with severe diarrhea prior to surgery. Parenteral nutrition may be needed in patients with weight loss and hypoproteinemia especially before major surgery. Niacin deficiency is common since the essential amino acid tryptophan is converted to serotonin in excessive amounts in carcinoid syndrome and needs to be addressed/monitored prior to surgery (*6*).

Massive acute release of neuroendocrine substances results in carcinoid crisis. Life-threatening hypotension, arrhythmias, bronchospasm, facial flushing, edema, metabolic acidosis, coma, confusion, and death characterize the crisis (*7*). Carcinoid crisis from bland embolization have been reported to occur peri- or 24 h after procedure (*8*). Carcinoid crisis can be precipitate by a number of environmental triggers such as stress, alcohol, amine containing food products, medications, and non-environmental triggers such as tumor manipulation during surgery, embolization, or chemotherapy (*9*). High pre-operative urinary 5-HIAA and carcinoid heart disease are significant risk factors for perioperative complications (*10*). Treatment of a carcinoid crisis includes fluid expansion and use of 50-500 μg IV octreotide. Current recommendation for treatment of carcinoid crisis with hypotension that develops during procedure is 500 μg IV bolus every 5 min until symptoms are controlled (*11*). Ketanserin (selective antagonist of the 5-hydroxytryptamine receptor 2, the α1-adrenoreceptor, and the H1-histamine receptor) 10 mg IV bolus can be used to block the actions of the offending mediators in carcinoid crisis (*12*). Avoidance of catecholamines in the treatment of hypotension is stressed because catecholamines may stimulate tumor cells to release even more serotonin.

Our patient presented with carcinoid crisis 24 h after bland embolization of liver metastases. Pre-procedure administration of octreotide remains the mainstay of preventive therapy for patients undergoing procedures relating to carcinoid tumors. For patients with extensive metastases, adjunct therapy with corticosteroids, cyproheptadine, and glucagon should be considered.

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Case Report

Cerebral sparganosis: A case report

Qiang Ou[1,2], Shujuan Li[2], Xunjia Cheng[1,*]

[1] *Department of Microbiology and Parasitology, Shanghai Medical College of Fudan University, Shanghai, China;*
[2] *Shanghai Public Health Clinical Center, Shanghai, China.*

Summary **Sparganosis is a rare parasitic infection in humans caused by a larval cestode of the genus Spirometra. Spargana invade muscle, subcutaneous tissue, the eye, urogenital, and abdominal viscera, but the central nervous system (CNS) is not a frequent site of invasion. Here, we describe an 18-year-old patient with cerebral sparganosis and review data from other cases reported in the medical literature.**

Keywords: Spirometra, cerebral sparganosis, albendazole

1. Introduction

Sparganosis is caused by a migrating tapeworm larva of the genus Spirometra. Spargana invade muscle, subcutaneous tissue, the eye, urogenital, and abdominal viscera, but the central nervous system (CNS) is not a frequent site of invasion. Rare but increasing reports have described cerebral sparganosis from many corners of the world (*1-3*), and recent developments in serologic and radiologic studies make diagnosis of CNS infection much easier than previously. We describe here a patient with sparganosis of the CNS and review data from other cases reported in the medical literature.

2. Case report

An 18-year-old female presented to the infectious department of Shanghai Public Health Clinical Center (SPHCC) with dizziness, headache, decreasing memory and reaction dullness, which had developed over the course of one month. Seizure occurred once during the course. On examination, her body temperature was 37.5°C, blood pressure was 120/80 mmHg and heart rate was 90/min. She had clear consciousness, but mental status was a little bit abnormal. Her reaction ability was dull and memory was decreased. Electrocardiogram (ECG) and X-ray checking for heart and lung was shown to normal signals. She had no nuchal rigidity with normal muscle strength and muscular tension. The signs of Kerning, Brudzinskin and Babinski were abnormal. Whole blood analysis revealed a leukocyte count of 6,500/mm^3, hemoglobin 11.2 g/dL and platelet count 210,000/mm^3. Data for her erythrocyte sedimentation rate, routine blood biochemistry and urine analysis were in the normal range. Anti-HIV antibodies, hepatitis B surface antigen, anti-hepatitis C virus antibodies, and rheumatoid factor were negative.

Lumbar puncture revealed high pressure in the cranial cavity, with a rapid dropping of cerebrospinal fluid (CSF). The leukocyte count and the level of glucose, protein and chloride were normal in CSF. There were no bacteria in Gram staining of the CSF sedimentation. However, the enzyme-linked immunosorbent assay for antisparganum antibody was strongly positive in blood and CSF confirmed by the Institute of Parasite of Chinese Center for Disease Control and Prevention. A cranial MRI was ordered. T1-weighted imaging (T1WI) showed that there was an irregular flake low signal focus in the white matter of left parietal lobe and its edge was vague (Figure 1A). T2-weighted imaging (T2WI) showed focus presented high signals (Figure 1B). Enhanced scans showed irregular enhancement focus and the range of enhancement was smaller than that of high signals observed in T2WI (Figure 1C). Diffusion weighted imaging showed tortuous beaded shape enhancement in the left corpus callosum and parietal lobe in sagittal plane (Figure 1D). Further inquiry into the medical history of the patient revealed she ate poorly cooked frog flesh five months ago.

The patient was finally diagnosed with cerebral

**Address correspondence to:*
Dr. Xunjia Cheng, Department of Microbiology and Parasitology, Shanghai Medical College of Fudan University, Shanghai 200032, China.
e-mail: xjcheng@shmu.edu.cn

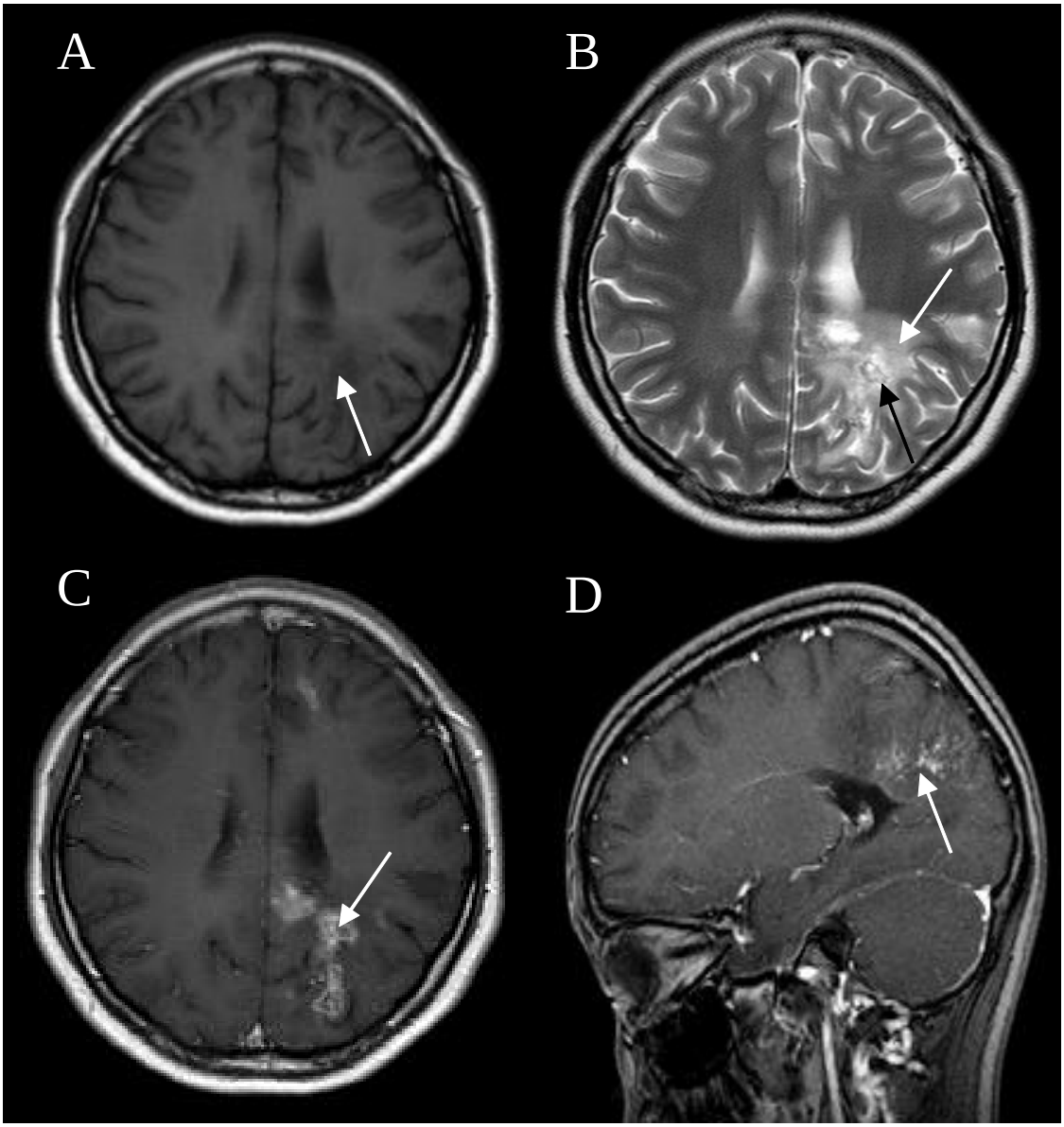

Figure 1. A cranial MRI taken during patient admission. (A) Irregular flake low signal focus in the white matter of left parietal lobe and its edge was vague (T1-weighted imaging, T1WI). **(B)** Focus presented high signals and the surrounding boundary was not clear (T2-weighted imaging, T2WI). **(C)** Enhanced scan showed irregular enhancement focus and the range of enhancement was smaller than that of abnormal high signals observated in T2WI. **(D)** Tortuous beaded shape enhancement in left corpus callosum and parietal lobe in sagittal plane (diffusion weighted imaging, DWI).

sparganosis. She rejected surgical operation and received abendazole (300 mg twice a day) pulsed sodium valproate therapy (200 mg twice a day). The course of treatment was 20 days. The patient was discharged 4 weeks later with no signs or symptoms. No neurological relapse was observed during a one-year follow-up.

3. Discussion

Sparganosis is a rare parasitic infection in humans caused by a larval cestode of the genus Spirometra. Human infection is uncommon and seems to occur accidentally by ingestion of polluted water containing cyclops in which tapeworm eggs mature into procercoid larvae, by ingestion of raw or inadequately cooked flesh of snakes or frogs, and by applying the flesh of an infected intermediate host as a poultice to a wound. The larvae can migrate from primary infection focus to a distant site, such as skin, skeletal muscle, orbital tissue, urogenital and abdominal viscera, and rarely to the CNS. The infective route in this case was probably through eating poorly cooked frog flesh five months ago. There were no other signs or symptoms resulting from sparganum detected other than the brain.

The clinical manifestations of cerebral sparganosis varied depending on the site of infection, including seizure, progressive headache, dizziness, consciousness disturbance and focal neurologic deficits such as hemiparesis, aphasia, and dysarthria. Among these,

seizure and headache were the most frequent symptoms and signs. Fever was rare. However, the clinical presentations of cerebral sparganosis were similar to some other slow progressive CNS diseases. Differential diagnosis such as other infectious granulomas, brain neoplastic lesions, and tuberculous meningitis should be done, especially when the lesions are not responding to treatment (*1*). The patient in this report complained of one month of dizziness, headache, decreasing memory and reaction dullness. Seizure occurred once during the course.

The final diagnosis depends on pathologic or immunologic examination results. ELISA test is a very sensitive test for the detection of sparganosis when the diagnosis is in doubt. In surgically proven cerebral sparganosis, both serum and CSF ELISA tests showed high sensitivity and a concordance rate in diagnosing cerebral sparganosis. The serologic diagnosis by means of ELISA could be a useful tool in the epidemiologic study of human sparganosis.

Computed tomographic (CT) scans and magnetic resonance imaging (MRI) can be very helpful in diagnosing cerebral sparganosis. Typical MRI patterns of cerebral sparganosis included abnormal enhancement such as a peripheral ring-type, tortuous beaded shape or a serpiginous tubular shape enhancement, and changes in location and shape of lesions in the follow-up MRI. Cong *et al*. (*4*) reported that the diagnostic accuracy rate for cerebral sparganosis at the first visit, after first imaging examination and after several follow-up MRI examinations were 0%, 11.8% and 28.6%, respectively. Song *et al*. (*5*) considered that the most characteristic finding was a tunnel sign on postcontrast MRI. The most common finding was bead-shaped enhancement. MRI is superior to CT in demonstrating the extent and number of lesions, except for punctate calcifications. Combined with clinical data and enzyme-linked immunosorbent assay, the preoperative diagnosis of cerebral sparganosis could be established on MR imaging.

The treatment of cerebral sparganosis included surgical operation and medicine. Surgical operations included stereotactic techniques and total removal of the lesion. Patients with no indications for operations can be treated with praziquantel or albendazole, but the therapeutic effect of surgical operation was better than medical treatment (*6*). Gao (*7*) thinks early detection and surgical removal of the parasite and the surrounding granuloma is the best option for its treatment. Our patient was treated with albendazole for 20 days and no neurological relapse was observed during a one-year follow-up. But we need to do further research on the long-term effects of medical treatment.

Cerebral sparganosis is extremely rare. However, the diagnosis of cerebral sparganosis should be considered if patients show seizure, progressive headache, dizziness and migrating granulomatous lesions in the area of epidemic sparganosis. We should inquire about medical history and take pathologic or immunologic examinations in order to exclude the disease.

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Case Report

Anisocoria in liver recipients during the perioperative period: Two case reports

Ryo Orii[1], Yasuhiko Sugawara[2,*], Masatoshi Makuuchi[3], Norihiro Kokudo[2], Yoshitsugu Yamada[1]

[1] *Department of Anesthesiology, Department of Surgery, Faculty of Medicine, The University of Tokyo, Tokyo, Japan;*
[2] *Artificial Organ & Transplantation Division, Department of Surgery, Faculty of Medicine, The University of Tokyo, Tokyo, Japan;*
[3] *Department of Surgery, Japanese Red Cross Hospital, Tokyo, Japan.*

Summary **Two cases of anisocoria that occurred just after the induction of general anesthesia for living donor liver transplantation are reported. Space-occupying lesions were not observed in brain computed tomography. Mydriasis was temporary in both cases, suggesting that the anisocoria was most likely related to Adie syndrome.**

Keywords: Living-related liver transplant, recipient, anisocoria

1. Introduction

Anisocoria during general anesthesia may indicate a serious neurologic condition. Proper diagnosis of unilateral mydriasis associated with general anesthesia is complicated by decreased responsiveness or unresponsiveness of the patient induced by the anesthetic agents.

Cerebrovascular diseases such as a ruptured aneurysm, cerebral trauma, or a mass lesion should be ruled out. Liver transplant recipients tend to bleed easily due to hepatic dysfunction and coagulation defects and thus have a high risk of brain hemorrhage in the perioperative period (*1*). Many cases also present with an altered state of consciousness due to hyperammonemia.

Treatment with mydriatic medications or eye disorders such as edema or Adie syndrome (*2*) are included in the differential diagnosis (*3*). We report two cases of temporary anisocoria in two liver recipients during the perioperative period.

2. Case reports

2.1. *Case 1*

A 57-year-old female had diabetes for the past 3 years, which was appropriately managed using insulin administration. The patient developed bronchial asthma 9 years ago and has been receiving inhalation treatment. In 1995, the patient developed liver dysfunction and hepatitis C. Interferon was administered for 6 months. The patient developed cirrhosis in 2000, and in 2005 it became difficult to control the ascites. Partial liver transplantation and Hassab's operation were scheduled for decompensated cirrhosis and esophageal varices. The patient's preoperative consciousness level was alert. Anisocoria was not observed in the preoperative examination.

Anesthesia was induced by intravenous administration of 100 μg fentanyl, 10 mg midazolam, and 10 mg vecuronium bromide, while the trachea was intubated *via* the oral route. Anesthesia induction proceeded smoothly. Anesthesia was maintained with air/oxygen/isoflurane; 40% oxygen in air and an end-tidal isoflurane concentration of 1.5 to 3.0 vol%. Fentanyl and vecuronium were added as needed. No significant cyclical fluctuation was observed. After tracheal intubation, pupil diameters of 4 mm on the right and 1 mm on the left were confirmed. The papillary light reflex was slight in both eyes. Considering the possibility of an intracerebral lesion, an urgent head computed tomography (CT) scan was performed under endotracheal intubation, which revealed no abnormal findings. After final evaluation by a neurosurgeon, surgery was continued. There were no changes in the pupil diameters during the surgery and no significant fluctuations in the circulatory dynamics, and thus the patient was returned to the intensive care unit (ICU) postoperatively. The duration of the operation was 15 h and 25 min, and the duration of anesthesia was 18 h and 10 min. A postoperative CT revealed no

*Address correspondence to:
Dr. Yasuhiko Sugawara, Artificial Organ & Transplantation Division, Department of Surgery, Faculty of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
e-mail: yasusuga-tky@umin.ac.jp

abnormalities and no neurologic abnormalities were observed. After recovery from anesthesia and removal of the endotracheal tube in the ICU, no neurologic abnormalities other than anisocoria were observed. The anisocoria gradually decreased and the pupils became symmetrical within 2 weeks after surgery.

2.2. *Case 2*

A 45-year-old male visited our hospital with a primary complaint of systemic fatigue and was hospitalized with a diagnosis of fulminant hepatic failure. Although his hepatic encephalopathy improved, his total bilirubin remained high. After hospitalization, frequent plasma exchange was required, and an auxiliary partial orthotopic liver transplantation was performed. Anisocoria was not present prior to the induction of anesthesia.

In the operating theater, the patient's level of consciousness was clear. General anesthesia was induced by intravenous administration of 200 μg fentanyl, 200 mg thiopental, 5 mg midazolam, and 10 mg vecuronium bromide, and maintained by air/oxygen/isoflurane. Neither hypoxia, hyperventilation, severe hypotension, nor hypertension were present. After the induction of general anesthesia, anisocoria was observed (pupil diameters of 4 mm on the right and 3 mm on the left). We performed an urgent head CT to check for cerebral lesions, but no organic lesions or neurologic abnormalities were observed. A neurosurgical consultation determined that the operation could continue.

The pupils became symmetrical 11 h after the induction of anesthesia. The duration of the operation was 15 h and 5 min and the duration of anesthesia was 16 h and 50 min. There was nothing specifically notable during surgery. There were no abnormal findings in the postoperative head CT. After recovery from the anesthesia and removal of the endotracheal tube in the ICU, no neurologic abnormalities were observed.

3. Discussion

Anisocoria during general anesthesia has been reported (*4-9*). The causes of anisocoria in these previously reported cases included administration of mydriasis-inducing drugs (peripheral mechanism involving parasympathetic postganglionic fibers) into the ocular or nasal regions (*6-8*) and interventions (*5,9*) in the neck or cranium areas. Kobayashi (*10*) reported a case of anisocoria that occurred in association with a hypertensive episode after anesthetic induction. Adie syndrome was subsequently diagnosed by a positive pilocarpine test.

A relationship between inhalation-induced analgesia and anisocoria has also been reported. Sobel (*11*) described differences in pupil size with an incidence of 0.4% with the use of halothane, 0.8% with cylopropane, and 58% with diethyl ether. For anesthesia with cylopropane and diethyl ether, the elevated levels of catecholamines liberated by these compounds are likely responsible for the pupil dilatation (*12*). The higher incidence of anisocoria with diethyl ether compared to cylopropane is related to the more pronounced parasympathomimetic effect of diethyl ether.

A perfusion disturbance or imbalance in the orbital area may also be related to anisocoria. Rempf (*13*) reported a case in which anisocoria occurred during general anesthesia for renal transplantation. Retinal hypoperfusion detected with cranial and orbital Doppler sonography is a plausible explanation for the anisocoria. Klein (*4*) reported a case of anisocoria with exophthalmus during anesthesia with enflurane. The authors related the symptoms to the position of the head being lowered by 10° and the resulting venous pooling in the orbital area.

In our cases, anisocoria was observed just after the induction of general anesthesia. No space-occupying lesions were observed in the brain CT and the mydriasis was temporary, suggesting that the anisocoria was most likely related to Adie syndrome. The main factors contributing to the manifestation of Adie syndrome may be parasympathetic dominance by anesthetics such as thiopental or isoflurane. Botulism, myasthenia gravis, and dyshidrosis are associated with increased vulnerability of the systemic cholinergic nerves (*14*). Autonomic neuropathies are common in diabetes, and liver diseases and are possibly induced by the autoantibody to autonomic structures. Peripheral somatic neuropathy (93%) and autonomic neuropathy (50%) were common in patients with end stage liver disease (*15*). It is possible that the vecuronium influenced the autonomic tone in our patients (*10*). Adie syndrome should be considered in the differential diagnosis of patients who develop anisocoria in the perioperative setting.

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