ISSN 1881-7815 Online ISSN 1881-7823

BST BioScience Trends

Volume 6, Number 6
December, 2012

IRCA BSSA

IACMHR

www.biosciencetrends.com

BST
BioScience Trends

ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

BioScience Trends is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA and Shandong University China-Japan Cooperation Center for Drug Discovery & Screening (SDU-DDSC).

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(This journal was partially supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science.)

Brief Report

DOI: 10.5582/bst.2012.v6.6.283

Quantitative proteomic study identified cathepsin B associated with doxorubicin-induced damage in H9c2 cardiomyocytes

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Summary **The study was performed to analyze the proteomic profiling of doxorubicin-treated H9c2 cardiomyocytes in order to identify novel protein biomarkers associated with doxorubicin-induced cardiomyopathy. The protein profiling of H9c2 cells in response to doxorubicin at an apoptosis-induced concentration of 0.5 μM were compared using iTRAQ analysis. Western-blot analysis was used to confirm differentially expressed proteins identified in the proteomic study. A total of 22 differently expressed proteins were identified in doxorubicin-treated H9c2 cells including 15 up-regulated and 7 down-regulated proteins. Gene Ontology (GO) analysis revealed that 10 altered proteins were enriched in the process of apoptosis. We further validated the expression of cathepsin B and its possible regulator nuclear factor kappa B (NF-κB) in H9c2 cells were increased during doxorubicin treatment using Western-blots. Differentially expressed proteins might provide clues to clarify novel mechanisms underlying doxorubicin-induced cardiomyopathy. Our results also suggest that increased cathepsin B expression might be associated with NF-κB up-regulation, and the exact mechanisms need to be clarified.**

Keywords: Doxorubicin, cardiomyopathy, proteomics, cathepsin B, nuclear factor kappa B

1. Introduction

Doxorubicin is one of the most effective chemo-therapeutic agents, and is indicated for many cancers including breast cancers, lung cancers, and ovary cancers. However, the optimal clinical use of this agent is limited by its major adverse effect of cardiomyopathy which leads to congestive heart failure, which would cause a mortality of approximately 50% (*1,2*). Extensive research has suggested several possible mechanisms for its cardiomyopathy including apoptosis, oxidative stress, inactivated cardiomyocyte specific genes, and altered molecular signaling (for example, MAPKs) (*3*). However, unfortunately, no effective treatment for established doxorubicin cardiomyopathy is available. Therefore, there is a great need to identify novel targets to protect the heart from doxorubicin damage.

Recent developments in proteomic technology provide an opportunity for the discovery of novel molecular pathways and biomarkers of drug-induced toxicity. Especially, in a recent proteomic study using the common two-dimensional electrophoresis (2-DE) and mass spectrometry technologies, Kumar *et al.* (*4*) identified several oxidative stress response-related proteins differently regulated in rat cardiomyocytes and heart tissues exposed to doxorubicin. Compared with the traditional proteomic methods such as 2-DE

Bao GY, Wang HZ, and Shang YJ contributed equally to this work.

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gel analysis followed by MS/MS, a stable-isotope labeled strategy iTRAQ-coupled 2-D LC-MS/MS approach takes advantage of higher detection sensitivity and quantitative accuracy, and holds the promise of effectively detecting a cellular protein profile in response to drugs (*5*).

Therefore, in this present study, we performed a differential proteomic analysis on doxorubicin-treated H9c2 cardiomyocytes using iTRAQ technology in order to identify novel biomarkers or potential targets for treatment of doxorubicin-induced cardiomyopathy.

2. Materials and Methods

2.1. *Cell culture and treatment*

Cardiomyocyte cell line H9c2 derived from embryonic rat heart by selective serial passages was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) with the original source as American Type Culture Collection (ATCC) (Manassas, VA, USA). H9c2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) with 10% fetal calf serum (Gibco) at 37°C with 5% CO_2. H9c2 cells were treated with doxorubicin (Sigma-Aldrich, St. Louis, MO, USA) at a clinically relevant concentration of 0.5 μM as described previously (*6*).

2.2. *Flow cytometric analysis of 0.5 μM of doxorubicin on apoptosis of H9c2 cells*

H9c2 cells without or with doxorubicin treatment at different time courses were collected and washed in PBS. After washing and staining with annexin V-FITC/propidium iodide (PI), the apoptosis rate of H9c2 cells were detected using flow cytometry as directed by an Annexin V-FITC/PI Kit (Nanjing Kaiji Biological Inc., Nanjing, China).

2.3. *Protein digestion and labeling with iTRAQ reagents*

Protein extraction at 48 h was performed as described previously (*7*). Briefly, H9c2 cells treated with and without doxorubicin for 48 h were dissolved in lysis buffer (7 M urea, 2 M thiourea, 65 mM DTT, 0.1 mM PMSF) and protein concentrations were determined using the 2D Quant Kit (GE Healthcare, Uppsala, Sweden). Protein trypsin digestion and iTRAQ labeling were performed in accordance with the manuscript's protocol (Applied Biosystems, Carlsbad, CA, USA). After reduction and alkylation, 100 μg protein of each sample were digested with trypsin and labeled with iTRAQ reagents as follows: H9c2 cells treated with doxorubicin, iTRAQ reagent 113; and H9c2 cells treated without doxorubicin, iTRAQ reagent 115. The labeled digests were then combined for peptide separation.

2.4. *Trypsin peptides fraction and mass spectrometry*

The mixed peptides were fractionated using strong cation exchange (SCX) chromatography into 10 fractions. The peptides in these fractions were further separated using a C18 column on a Tempo LC nano-flow system, and spotted on stainless steel target plates using a MALDI spotting system (Applied Biosystems). Mass spectrometer 4800 MALDI TOF/TOF Analyzer (Applied Biosystems) was used to perform tandem mass spectrometry acquisition in the positive ion mode. Only the most intense ion signals with S/N above 40 and excluding common trypsin autolysis peaks were selected as precursors for MS/MS acquisition. The MS/MS data were searched using the MASCOT Database search engine (Matrix Science, London, UK) embedded into GPS-Explorer Software (Applied Biosystems) against the Swiss-Prot database with the following search parameters: peptide tol: 0.3 Da, MSMS tol: 0.6 Da, variable modification: oxidation (M), fixed modification: carboxymethyl (C). Proteins were identified with at least a 95% confidence level, and relative quantification of proteins was based on the ratio of peak areas of m/z 113 and 115 from the MS/MS spectra.

2.5. *Gene ontology (GO) analysis*

GO analysis was performed with the GSEABase package from the R statistical platform (*http://www.r-project.org*).

2.6. *Western-blot*

The iTRAQ analysis has been performed only once in this study, to confirm the expression variation of cathepsin B in H9c2 cells during doxorubicin treatment, and we further performed a Western-blot experiment in another independent series of samples, which is not the same as those used in the proteomic study. Whole cell extracts and nuclear protein extracts were prepared using a protein extraction kit (Beyotime Bio-tech, Haimen, China) according to the manufacturer's protocol. Thirty μg of the extracted protein samples were separated using 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Primary antibody incubation was carried out overnight at 4°C using mouse monoclonal antibodies against cathepsin B (1: 1000, Abcam, Cambridge, UK) and nuclear factor kappa B (NF-κB) p50 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then probed with the corresponding IgG-HRP. Protein bands were visualized using enhanced chemiluminescence reagents (Milipore, Billerica, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

3. Results and Discussion

As seen in Figure 1, our data indicates 0.5 μM doxorubicin exerted a considerable apoptosis-inducing effect on H9c2 cardiomyocytes in a time-dependent manner. We then applied an iTRAQ-based proteomic strategy to assess differential expression of H9c2 cells affected by doxorubicin at 48 h on the proteomic level. Proteins giving tryptic peptides with a 113/115 ratio ≥ 2 were classified as up-regulated and a ratio ≤ 0.5 as down-regulated. We confidently identified a total of 212 non-redundant proteins in the iTRAQ analysis. Twenty-two differentially expressed unique proteins were revealed including 15 up-regulated and 7 down-regulated proteins. The protein and gene names, database ID, molecular weight, isoelectric point (pI), and fold changes of identified proteins are listed in Tables 1 and 2. GO analysis on apoptosis related biological process revealed that 3 up-regulated genes (*Anxa1, Cryab, Lmna*) and 6 down-regulated genes (*Hspa5, Phb, Hspb1, Hspd1, Vdac1, Pdia3*) were enriched in the process of apoptosis (Figure 2).

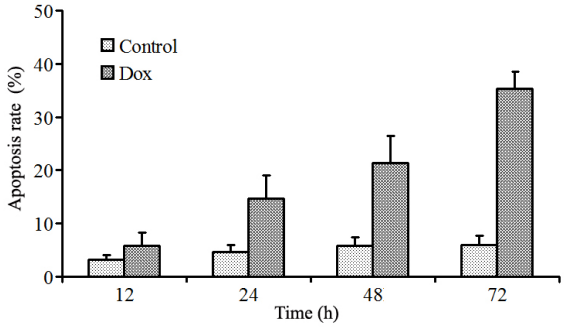

Figure 1. Flow cytometric analysis of 0.5 μM doxorubicin on apoptosis of H9c2 cells.

Of the differentially expressed proteins, cathepsin B (with two matched peptides: GENHCGIESEIVAGIPR and SGVYKHEAGDVMGGHAIR, peptide coverage of 11%), which had not been associated with doxorubicin-induced cardiac toxicity identified in previous studies, was selected to be further confirmed by Western-blot analysis. As seen in Figure 3, the expression of cathepsin B in H9c2 cells was increased during doxorubicin treatment and peaked at 48 h, which was in accordance with the proteomic results. Furthermore, expression of possible cathepsin B regulator NF-κB p50 protein in the nucleus increased after doxorubicin induction, which is consistent with the variation of cathepsin B.

In the current study, we quantitatively compared the global proteomic profiling of rat cardiomyocyte H9c2 cells with and without doxorubicin addition at a clinical relevant concentration, which exhibited an apoptosis-inducing effect, but not significant cellular damage in H9c2 cells (*6,8*). Our proteomic analysis revealed a series of proteins varied in cardiomyocytes after doxorubicin treatment for 48 h, in which 10 differentially regulated proteins were enriched in the biological process of apoptosis, which have been

Table 1. List of the up-regulated proteins identified in the iTRAQ experiments

No.	Accession number	Protein name	Gene	Molecular weight (Da)	pI	Ratio
1	P23928	α-Crystallin B chain	*Cryab*	20,088	6.76	2.3
2	P07150	Annexin A1	*Anxa1*	38,829	6.97	3.1
3	P55260	Annexin A4	*Anxa4*	35,848	5.30	2.1
4	P04639	Apolipoprotein A-I	*Apoa1*	30,062	5.52	2.6
5	P15999	ATP synthase subunit α, mitochondrial	*Atp5a1*	59,753	9.22	4.4
6	P00787	Cathepsin B	*Ctsb*	37,469	5.36	3.6
7	P48675	Desmin	*Des*	53,456	5.21	2.2
8	P04764	α-Enolase	*Eno1*	47,127	6.16	3.1
9	O35814	Heat shock cognate 71 kDa protein	*Stip1*	70,871	5.37	2.0
10	P48679	Lamin A/C	*Lmna*	72,418	6.41	2.7
11	Q9ER34	Aconitate hydratase, mitochondrial	*Aco2*	85,433	7.87	3.7
12	Q68FX0	Isocitrate dehydrogenase subunit β, mitochondrial	*Idh3B*	42,353	8.89	3.5
13	O35814	Stress-induced-phosphoprotein 1	*Stip1*	62,570	6.40	6.2
14	P48721	Stress-70 protein, mitochondrial	*Hspa9*	73,857	5.97	2.3
15	A9YUA5	Cardiac troponin T2	*Tnnt2*	34,452	5.19	3.1

Table 2. List of the down-regulated proteins identified in the iTRAQ experiments

No.	Accession number	Protein name	Gene	Molecular weight (Da)	pI	Ratio
1	P63039	60 kDa heat shock protein, mitochondrial	*Hspd1*	60,955	5.91	0.32
2	P06761	78 kDa glucose-regulated protein	*Hspa5*	72,347	5.07	0.21
3	P60711	Actin, cytoplasmic 1	*Actb*	41,737	5.29	0.14
4	P11598	Protein disulfide-isomerase A3	*Pdia3*	56,623	5.88	0.19
5	P42930	Heat shock protein β-1	*Hspb1*	22,893	6.12	0.41
6	P67779	Prohibitin	*Phb*	29,820	5.57	0.31
7	Q9Z2L0	Voltage-dependent anion-selective channel protein 1	*Vdac1*	30,756	8.62	0.11

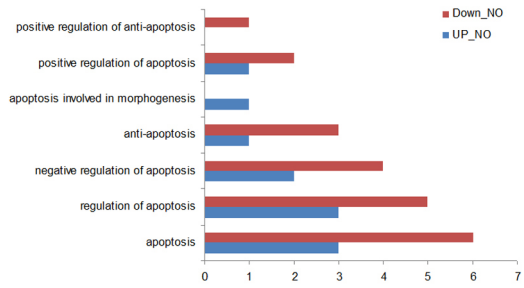

Figure 2. GO annotation on the apoptosis biological process on up-regulated proteins (UP) and down-regulated proteins (Down) identified in the proteomic experiments.

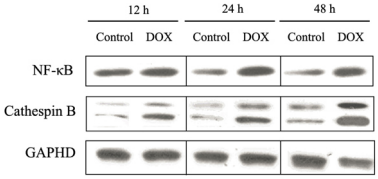

Figure 3. Western blot analysis detected the level of cathepsin B and nuclear NF-κB p50 expression in H9c2 cells during doxorubicin (DOX) treatment.

proved to be closely associated with doxorubicin-induced cardiac toxicity in previous studies. Among these aberrantly regulated proteins, several proteins such as cardiac troponin-T, prohibitin, and HSP60 have already been described to be involved in the generation of doxorubicin-induced cardiomyopathy (*6,9-11*).

We selected cathepsin B as a novel candidate doxorubicin-associated protein for the further validation and function study for the following reasons. First, this protein in H9c2 cells increased significantly in the proteomic study. Secondly, the role of cathepsin B in the pathogenesis of doxorubicin-induced cardiomyopathy has not been reported. However, the evidence in cancer cells indicates that cathepsin B is a crucial component in doxorubicin-induced apoptosis. For example, Bien *et al*. (*12*) found that cathepsin B could mediate doxorubicin-induced cell death by the mechanisms of loss of activating caspase 3, cleavaging PARP and inhibiting cdk1 *et al*. in tumor cells. Our Western-blot analysis indicated that doxorubicin induced a time-dependent up-regulation of cathepsin B protein increase in H9c2 cells, which is in accordance with those of our proteomic analysis. This finding suggests cathepsin B is a key regulatory component in doxorubicin-induced cardiomyopathy, which might be similar to its modulatory role in doxorubicin-induced tumor cell death. Bien *et al*. (*12*) also found that doxorubicin induces cathepsin B expression and activity *via* NF-κB in tumor cells, and Li *et al*. (*13*) found that expression of NF-κB p50 protein in the nucleus and NF-κB binding activity increased significantly in cardiomyopathy induced by adriamycin. In this study, we also demonstrate that doxorubicin induced cathepsin B expression was accompanied by up-regualtion of NF-κB. Recently, Qiao *et al*. (*14*) also found a co-occurrence of NF-κB activation and cathepsin B up-regulation in delayed anesthetic preconditioning protection against myocardial infarction. Together with the data of the previous studies, our findings suggest that up-regulation of cathepsin B by doxorubicin in cardiomycytes might be associated closely with NF-κB, and the exact mechanisms still deserve further investigation.

Cathepsin B is a prominent lysosomal protease, and plays an important role in the apoptosis process and myocardial damage (*15*). Tsuchida *et al*. (*16*) demonstrated that cathepsin B was involved in degradation of myofibrillar proteins in myocardial infarction. Ge *et al*. (*17*) also found elevated cathepsin B expression might modulate myocardial apoptosis in patients with dilated cardiomyopathy. Therefore, we proposed that cathepsin B could be increased by doxorubicin, and act as a potent apoptosis inducer during the following myocardial toxicity. However, the exact mechanisms of cathepsin B in doxorubicin induced cardiomyopathy remain not fully elucidated, and deserve further investigation.

Considering its role in doxorubicin-induced tumor cell death, it has been proposed that increasing cathepsin B expression could be a novel strategy to modify susceptibility towards doxorubicin (*18*). But our results indicated that up-regulated cathepsin B would also aggravate cardiomyopathy. Therefore, when cathepsin B is considered as an attractive target for sensitizing chemotherapy in cancer, its potential cardiac toxicity must be evaluated carefully first.

In conclusion, in this present study, using iTRAQ-based proteomic methods, we identify a panel of differentially expression proteins related to doxorubicin treatment in cardiomyocytes, which might provide clues to clarify novel mechanisms underlying doxorubicin-induced cardiomyopathy. Among these proteins, cathepsin B expression was further confirmed by Western-blots, and our results also suggest that increased cathepsin B expression might be associated with the up-regualtion of NF-κB in doxorubicin-induced cardiomyopathy.

Acknowledgements

This research was supported by grants from National Science Foundation of China (30972730, 30772017), and Shanghai Municipal Commission for Science and Technology (09JC1405400, 11JC1410902).

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(*Received September 4, 2012; Revised November 18, 2012; Accepted December 18, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.6.288

Prevalence and correlates of alcohol use and subsequent sexual activity among adult males in a rural community of ethnic minorities in Yunnan Province, China

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Summary **This community-based cross-sectional study examined alcohol use and HIV risks among a sample of predominantly ethnic males in Yunnan Province, China. Information about alcohol use, sexual behavior, sex after drinking, and HIV infection was collected using face-to-face interviews and blood testing. Out of 497 potential male participants, 382 males agreed to participate in this study. Of these males, 70% were ethnic minorities, 74.1% were currently married, 95.5% were sexually experienced, 27.5% had used drugs, and 6% were HIV-infected. Over 81% were current drinkers and 55.7% started drinking before the age of 18. Among current drinkers, 44.5% drank daily and 31.9% had drunk heavily in the past 30 days. Baijiu (a Chinese liquor distilled from sorghum with an ethanol content of at least 40%) was the preferred drink of choice. Excessive alcohol use was associated with being an ethnic Jingpo (OR = 1.96), being a smoker (OR = 2.09) and having multiple lifetime sex partners (OR = 1.55). Over 21% reported having ever engaged in sex after drinking. Those who were aged 26 to 35 (OR = 3.80), started drinking before age 18 (OR = 2.14), who were heavy drinkers (OR = 1.99), or who had ever used drugs (OR = 2.00) were more likely to have ever engaged in sex after drinking. Health education programs for alcohol abuse and unwanted outcomes, particularly the risk of HIV, are urgently needed for ethnic males in Yunnan.**

Keywords: Alcohol use, sex, drug use, HIV, minority

1. Introduction

Alcohol consumption has become the world's third largest risk factor for disease and disability (*1*). An estimated 3.8% of all global deaths and 4.6% of global disability-adjusted life-years are attributable to alcohol, more than those caused by HIV/AIDS, violence, or tuberculosis (*2-4*). Sexual risk practices due to alcohol use and/or abuse have been studied extensively in many countries but not in China (*5-9*). The limited body of research investigating the association between alcohol use and sexual risk practices in China has mainly focused on specific populations such as drug users, migrants, female sex workers, and men having sex with men (MSM) in metropolitan and coastal areas (*10-18*). Little is known about alcohol use and its association with sexual risk practices and HIV infection among Chinese ethnic minorities, many of which have been disproportionately affected by the HIV epidemic (*19*).

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This is particularly true in Yunnan Province, a major epicenter of the drug and HIV epidemics in China (*20-22*). To address this significant gap, a pilot, cross-sectional study of adult males belonging to ethnic minorities in rural Yunnan Province was conducted in order to examine the prevalence and correlates of alcohol use and subsequent sexual activity as well as HIV status.

2. Methods

2.1. *Study site and participants*

This study was conducted in Dehong Dai and Jingpo Autonomous Prefecture (which borders Myanmar) in the west of Yunnan Province, where the first HIV outbreak in China was reported (*23*). Half of the 1.2 million permanent residents in this prefecture are ethnic minorities, the two largest groups of which are the Dai and Jingpo. The present study was conducted in 2011 in a rural community with 10 villages and ethnic minorities. A roster of registered male residents in these 10 villages was obtained from local authorities as a sampling frame. All permanent male residents aged 16 to 55 years who were able to provide informed consent were recruited for the study. In the Dai and Jingpo cultures, 16 is the age of consent. The upper age limit was set at 55 because life expectancy in this region is lower than the national average.

2.2. *Data collection*

A paper-pencil interview was conducted face-to-face by trained local public health workers in private settings – mostly participants' homes – using a four-part structured questionnaire covering (*i*) demographic characteristics, (*ii*) alcohol use, (*iii*) smoking and other drug use, and (*iv*) sexual behavior. Participants received a small incentive equivalent to US$5 as compensation for their time. All participants were assured of the confidentiality of their information on the informed consent form and throughout the interview. This study was approved by the Institutional Review Board (IRB) of Fudan University, Shanghai, China.

2.3. *Measurements*

2.3.1. *Demographic characteristics*

Due to the profile of the study population (rural ethnic minorities with low rates of literacy) data were collected on just four demographic characteristics – (*i*) age, (*ii*) education, (*iii*) ethnicity and (*iv*) marital status – in order to minimize stigma.

2.3.2. *Alcohol use*

A drinker was defined as an individual who had drunk at least once a month for more than one year. Two questions in the questionnaire were used to measure the prevalence of drinking: (*i*) "Have you ever drunk alcohol in your life?" and (*ii*) "Did you drink alcohol in the past 30 days?" If a respondent answered "yes" to the first question, then this person was defined as an ever drinker. If a respondent answered "yes" to both questions, then this person was defined as a current drinker.

Participants were asked about the types and quantities of beer and baijiu (a Chinese liquor distilled from sorghum) or other spirits consumed during a typical occasion such as lunch or dinner in order to measure alcohol (ethanol) consumption. To calculate the absolute amount of alcohol consumed during a typical occasion, an ethanol content of 4% was assumed for beer and 40% was assumed for baijiu or other spirits. The quantity of alcohol consumed was then calculated by multiplying the number of milliliters consumed by the percentage of ethanol contained in each type of beverage. If a drinker consumed both types of beverages during the same occasion, their absolute alcohol consumption was added together. Using guidelines from the World Health Organization, a low level of alcohol (ethanol) consumption was defined as drinking one to 40 grams per typical occasion, a medium level as 41 to 60 grams per typical occasion, a high level as 61 to 100 grams per typical occasion, and a very high level as 100+ grams per typical occasion (*24*).

2.3.3. *Smoking and other drug use*

A current smoker was defined as an individual who had smoked at least 100 cigarettes over his lifetime (*25*) and who had smoked during the past month. Lifetime illicit drug use including opium, heroin, ephedrine, ketamine, or ecstasy, and drug injection behaviors such as needle sharing were also assessed.

2.3.4. *Sexual behavior*

Lifetime sexual experience including the number of one's sexual partners was assessed. For alcohol users, lifetime sexual activity after drinking was assessed by specific type of sexual behavior (such as commercial sex or casual sex) as well as the rate of condom use. "Sexual activity after drinking" was defined as any sexual intercourse occurring subsequent to drinking.

2.3.5. *HIV testing*

All participants except those who were already confirmed as HIV-infected before this study were invited to and agreed to undergo HIV testing. Venous blood was collected by professional nurses using disposable sterile needles and tubes, stored in a cold box, and transported to the laboratory within four hours. Blood specimens were screened for HIV

Table 1. Characteristics of study participants

Variables	Jingpo (n = 225) No. (%)	Other (n = 157) No. (%)	Total (n = 382) No. (%)
Current age (years) (χ^2 = 6.85, p = 0.07)			
16-25	47 (20.9)	46 (29.3)	93 (24.3)
26-35	66 (29.3)	35 (22.3)	101 (26.4)
36-45	61 (27.1)	50 (31.8)	111 (29.1)
46-55	51 (22.7)	26 (16.6)	77 (20.2)
Education (years) (χ^2 = 10.31, p = 0.02)			
0 (Illiterate)	25 (11.1)	5 (3.2)	30 (7.9)
1-6	99 (44.0)	72 (45.9)	171 (44.8)
7-9	79 (35.1)	55 (35.0)	134 (35.1)
≥ 10	22 (9.8)	25 (15.9)	47 (12.3)
Marital status (χ^2 = 3.84, p = 0.14)			
Never married	47 (20.9)	40 (25.5)	87 (22.8)
Currently married	168 (74.7)	115 (73.2)	283 (74.1)
Divorced or widowed	10 (4.4)	2 (1.3)	12 (3.1)
Current smoker (χ^2 = 6.45, p = 0.01)			
Yes	184 (81.8)	111 (70.7)	295 (77.2)
No	41 (18.2)	46 (29.3)	87 (22.8)
Smoking in the past 30 days (cigarettes per day) (χ^2 = 2.05, p = 0.15)			
1-19	75 (40.8)	36 (32.4)	111 (37.6)
≥ 20	109 (59.2)	75 (67.6)	184 (62.4)
Ever used drugs (χ^2 = 26.63, p < 0.001)			
Yes	84 (37.3)	21 (13.4)	105 (27.5)
No	141 (62.7)	136 (86.5)	277 (72.5)
Ever injected drugs (p = 0.02[a])			
Yes	18 (21.4)	0 (0)	18 (17.1)
No	66 (78.6)	21 (100)	87 (82.9)
Ever shared needles to inject drugs			
Yes	15 (83.3)	0 (0)	15 (83.3)
No	3 (16.7)	0 (0)	3 (16.7)
Sexually experienced (χ^2 = 1.03, p = 0.31)			
Yes	217 (96.4)	148 (94.3)	365 (95.5)
No	8 (3.6)	9 (5.7)	17 (4.5)
Number of lifetime sexual partners (χ^2 = 9.88, p = 0.007)			
0-1	62 (27.6)	67 (42.7)	129 (33.8)
2-4	87 (38.7)	52 (33.1)	139 (36.4)
≥ 5	76 (33.8)	38 (24.2)	114 (29.8)
HIV-infected (χ^2 = 10.62, p = 0.001)			
Yes	21 (9.3)	2 (1.3)	23 (6.0)
No	204 (90.7)	155 (98.7)	359 (94.0)

[a] Fisher's exact test.

infection using an enzyme-linked immunosorbent assay (ELISA) (Kehua Biotech, China). Any samples that screened positive for HIV were confirmed by a Western blot assay (HIV BLOT 2.2; Genelabs Diagnostics, Singapore). All tests were performed according to the manufacturer's instructions. Participants were informed that they could obtain their test results by calling a designated telephone number and providing their identification number and name. If a participant tested positive for HIV, the participant would be counseled and registered with the national HIV/AIDS case reporting and surveillance system. The individual would also undergo routine follow-up CD4+ T-cells counts and, if appropriate, free antiretroviral treatment following national guidelines.

2.4. *Statistical analysis*

Data were analyzed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). In addition to descriptive analyses, tests of associations between two categorical variables were based on the chi-square test or Fisher's exact test, where appropriate. A multivariate ordinal logistic regression analysis adjusting for potential confounding variables was performed to examine correlates of a high risk of ethanol consumption during a typical occasion. Univariate and multivariate logistic regression analyses were also performed to explore correlates of subsequent sex after drinking among drinkers who had drunk in the past 30 days (*i.e.*, current drinkers). Respective odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated. A significance level of 0.05 was used for all tests.

3. Results

3.1. *Socio-demographic characteristics*

A total of 497 male residents were eligible for this study, of whom 115 (23.1%) declined to participate. Reasons for non-participation were relocation elsewhere (73.0% or 84/115) and incarceration for drug use (26.1% or 30/115) or other crimes (0.9% or 1/115). Among the 382 participants, 113 (29.6%) were Han (China's predominant ethnic group), 225 (58.9%) were Jingpo, 29 (7.6%) were Dai, and 15 (3.9%) belonged

to another ethnic minority. Of the participants, 7.9% were illiterate and 44.8% had only a primary school education, and 22.8% were never married. The mean age of participants was 35.3 years (S.D. = 10.9). Ethnic Jingpo participants did not significantly differ from other participants in terms of age and marital status but were significantly less educated (Table 1).

3.2. *Alcohol use: Lifetime and in the past 30 days*

Table 2 presents detailed information about alcohol use among participants. Over 90% were defined as ever drinkers, of whom 192 (55.7%) started drinking before age 18 and 310 (89.8%) were current drinkers (*i.e.*, drank in the past 30 days). Among current drinkers, 44.5% drank daily, 13.5% often drank in the morning, 48.4% drank only baijiu, 19.0% drank only beer, and 32.6% drank both baijiu and beer. Compared to other participants, Jingpo participants were more likely to be ever drinkers, to be current drinkers, to have drunk daily in the past 30 days, and to often drink in the morning. Surprisingly, Jingpo participants were less likely to start drinking at an earlier age than other participants.

3.3. *Prevalence and correlates of high or very high levels of alcohol consumption during a typical occasion in the past 30 days*

Nearly 50% of participants had consumed low or medium levels of alcohol during a typical occasion in the past 30 days, while approximately 32% consumed high or very high levels. Bivariate chi-square analysis showed that Jingpo participants were more likely to have consumed high or very high levels of alcohol in the past 30 days. After controlling for potential confounding variables, multivariate ordinal logistic regression analysis indicated that the level of alcohol consumption in the past 30 days was positively associated with being Jingpo (OR = 1.96, 95% CI: 1.29-2.97) and a current smoker (OR = 2.09, 95% CI:

Table 2. Alcohol use among study participants

Variables	Jingpo (n = 225) No. (%)	Other (n = 157) No. (%)	Total (n = 382) No. (%)
Ever drinker (χ^2 = 11.86, p = 0.001)			
Yes	213 (94.7)	132 (84.1)	345 (90.3)
No	12 (5.3)	25 (15.9)	37 (9.7)
Age (years) of drinking initiation among ever drinkers (χ^2 = 15.29, p < 0.001)			
≤ 18	111 (47.4)	91 (68.9)	192 (55.7)
≥ 19	112 (52.6)	41 (31.1)	153 (44.3)
Current drinker (*i.e.*, had drunk in the past 30 days) among ever drinkers (χ^2 = 0.35, p = 0.55)			
Yes	193 (90.6)	117 (88.6)	310 (89.8)
No	20 (9.4)	15 (11.4)	35 (10.2)
Frequency of drinking in the past 30 days among current drinkers (χ^2 = 15.84, p < 0.001)			
Daily or nearly daily	94 (48.7)	44 (37.6)	138 (44.5)
Often	50 (25.9)	18 (15.4)	68 (21.9)
Occasionally	49 (25.8)	55 (47.0)	104 (35.5)
Often drinking in the morning in the past 30 days (χ^2 = 11.38, p = 0.001)			
Yes	36 (18.7)	6 (5.1)	42 (13.5)
No	157 (81.3)	111 (94.9)	268 (86.5)
Type of alcoholic beverage consumed in the past 30 days (χ^2 = 3.64, p = 0.06)			
Baijiu only	100 (51.8)	50 (42.7)	150 (48.4)
Beer only	31 (16.1)	28 (23.9)	59 (19.0)
Both	62 (32.1)	39 (33.3)	101 (32.6)
Level of ethanol consumption during a typical occasion in the past 30 days (χ^2 = 15.76, p < 0.001)			
None	32 (14.2)	40 (25.5)	72 (18.8)
Low or medium	105 (46.7)	83 (52.9)	188 (49.2)
High or very high	88 (39.1)	34 (21.7)	122 (31.9)
Attended clinic due to drinking in the past year (χ^2 = 0.44, p = 0.51)			
Yes	30 (15.5)	15 (12.8)	45 (14.5)
No	163 (84.5)	102 (87.2)	265 (85.5)
Lifetime experience of sex after drinking (χ^2 = 2.27, p = 0.13)			
Yes	47 (24.4)	20 (17.1)	67 (21.6)
No	146 (75.6)	97 (82.9)	243 (78.4)
Lifetime experience of commercial sex after drinking (p = 0.035[a])			
Yes	9 (19.1)	0 (0)	9 (13.4)
No	38 (80.9)	20 (100)	58 (86.6)
Lifetime experience of casual sex after drinking (χ^2 = 6.74, p = 0.009)			
Yes	20 (42.6)	2 (10.0)	22 (32.8)
No	27 (57.4)	18 (90.0)	45 (67.2)
Condom use during sex after drinking (χ^2 = 2.26, p = 0.32)			
Never	32 (68.1)	10 (50.0)	42 (62.7)
Sometimes	9 (19.1)	7 (35.0)	16 (23.9)
Always	6 (12.8)	3 (15.0)	9 (13.4)

[a] Fisher's exact test.

Table 3. Prevalence and correlates of alcohol consumption level during a typical occasion in the past 30 days among study participants (*n* = 382)

Variables	None (*n* = 72), No. (%)*	Low or medium (*n* = 188), No. (%)*	High or very High (*n* = 122), No. (%)*	OR (95% CI)[a,b]	*p*[b]
Current age (years)					
16-25	17 (18.3)	59 (63.4)	17 (18.3)	1.00	
26-35	13 (12.9)	52 (51.5)	36 (35.6)	1.47 (0.80-2.70)	0.220
36-45	27 (24.3)	48 (43.2)	36 (32.4)	1.17 (0.61-2.25)	0.636
46-55	15 (19.5)	29 (37.7)	33 (42.9)	1.92 (0.93-3.96)	0.077
Ethnicity					
Jingpo	32 (14.2)	105 (46.7)	88 (39.1)	1.96 (1.29-2.97)	0.002
Other	40 (25.5)	83 (52.9)	34 (21.7)	1.00	
Education (years)					
≤ 6	43 (21.4)	90 (44.8)	68 (33.8)	0.83 (0.55-1.24)	0.365
≥ 7	29 (16.0)	98 (54.1)	54 (29.8)	1.00	
Marital status					
Never married	18 (20.7)	47 (54.0)	22 (25.3)	1.00	
Ever married	54 (18.3)	141 (47.8)	100 (33.9)	0.96 (0.55-1.70)	0.900
Current smoker					
Yes	40 (13.6)	154 (52.2)	101 (34.2)	2.09 (1.28-3.40)	0.003
No	32 (36.8)	34 (39.1)	21 (24.1)	1.00	
Ever used drugs					
Yes	15 (14.3)	51 (48.6)	39 (37.1)	0.97 (0.59-1.60)	0.907
No	57 (20.6)	137 (49.5)	83 (30.0)	1.00	
Multiple lifetime sex partners					
Yes	34 (26.4)	61 (47.3)	34 (26.4)	1.55 (0.99-2.42)	0.055
No	18 (12.9)	77 (55.4)	44 (31.7)	1.00	
HIV-infected					
Yes	5 (21.7)	9 (39.1)	9 (39.1)	0.71 (0.30-1.70)	0.443
No	67 (18.7)	179 (49.9)	113 (31.5)	1.00	

[a] OR, odds ratio; CI, confidence interval. [b] Obtained from multivariate ordinal logistic regression analysis adjusting for potential confounding variables listed in the table. * Proportions were calculated in the row.

1.28-3.40) but was not significantly associated with age, education level, marital status, drug use, multiple lifetime sex partners, or HIV infection status (Table 3).

3.4. *Smoking and other drug use*

As shown in Table 1, 77.2% of study participants were current smokers, and the proportion of current smokers was significantly higher among Jingpo than among other ethnic groups (81.8% *vs*. 70.7%, $\chi^2 = 6.45$, $p = 0.01$). Among current smokers, a majority (62.4%) smoked more than 20 cigarettes per day (CPD) during the past 30 days, including 59.2% of Jingpo and 67.6% of other ethnic groups, though there was no statistical difference.

More than one-quarter (27.5%) of participants were drug users, of whom 17.1% had injected drugs. Drug use was much more common among Jingpo than other ethnic groups (37.3% *vs*. 13.4%, $\chi^2 = 26.63$, $p < 0.001$). Roughly one-fifth (21.4%) of Jingpo drug users had ever injected drugs, 83.3% of whom had ever shared a needle when doing so. None of the drug users from other ethnic groups had ever used injection drugs (Table 1).

3.5. *Sexual behavior and HIV infection*

Among the study participants, 95.5% were sexually experienced and 66.2% had had two or more sexual partners in their lifetime (*i.e.*, multiple lifetime sex partners). Of the Jingpo participants, 72.4% had had multiple lifetime sex partners, which was significantly higher than the proportion in other ethnic groups (57.3%). The prevalence of HIV infection was 6% overall, 9.3% among Jingpo, and 1.3% among other ethnic groups. HIV prevalence was significantly higher among the Jingpo than among other ethnic groups (Table 1).

3.6. *Sexual activity after drinking*

Among the 310 current drinkers, 67 (21.6%) had ever engaged in sex after drinking. Of those, 13.4% (9/67) had engaged in commercial sex after drinking, 32.8% (22/67) had engaged in casual sex after drinking, and 62.7% (42/67) had never used condoms during sex after drinking. Both commercial sex and casual sex after drinking were more common among Jingpo than among other ethnic groups (Table 2).

Univariate logistic regression analyses showed that lifetime sexual activity after drinking was significantly correlated with age, age of alcohol initiation, alcohol consumption, drug use, and HIV infection status. After controlling for potential confounding variables, multivariate logistic regression analysis indicated that those who were age 26 to 35 (OR = 3.80, 95% CI: 1.38-10.52, $p = 0.01$), started drinking before age 18 (OR = 2.14, 95% CI: 1.08-4.22, $p = 0.03$), consumed high or very high levels of alcohol during a typical occasion in the past 30 days (OR = 1.99, 95% CI: 1.05-3.76, $p = 0.04$), or had ever used drugs (OR = 2.00, 95% CI: 1.00-4.01, $p = 0.05$) were more likely to have ever engaged in

Table 4. Prevalence and correlates of lifetime sexual activity after drinking among current drinkers (*n* = 310)

Variables	Prevalence of subsequent sex after drinking (%)	OR (95%CI)[a,b]	p^b	OR (95% CI)[c]	p^c
Current age (years)					
16-25	19.7	1.93 (0.73-5.09)	0.183	3.29 (0.97-11.13)	0.055
26-35	34.1	4.06 (1.65-10.01)	0.002	3.80 (1.38-10.52)	0.01
36-45	17.9	1.71 (0.65-4.48)	0.277	1.77 (0.63-4.96)	0.280
46-55	11.3	1.00		1.00	
Ethnicity					
Jingpo	24.4	1.56 (0.87-2.80)	0.134	1.12 (0.57-2.21)	0.738
Other	17.1	1.00		1.00	
Education (years)					
≤ 6	22.2	1.07 (0.62-1.83)	0.814	0.97 (0.52-1.81)	0.931
≥ 7	21.1	1.00		1.00	
Marital status					
Never married	21.7	1.00		1.00	
Ever married	21.6	0.99 (0.52-1.90)	0.977	1.65 (0.74-3.67)	0.219
Current smoker					
Yes	23.1	1.77 (0.79-3.95)	0.165	1.50 (0.62-3.63)	0.368
No	14.5	1.00		1.00	
Frequency of drinking in the past 30 days					
Daily or nearly daily	24.6	1.94 (0.99-3.79)	0.053	1.45 (0.66-3.19)	0.352
Often	26.5	2.14 (0.99-4.60)	0.053	1.81 (0.77-4.30)	0.176
Occasionally	14.4	1.00		1.00	
Age (years) of drinking initiation					
≤ 18	27.4	2.31 (1.28-4.15)	0.005	2.14 (1.08-4.22)	0.03
≥ 19	14.1	1.00		1.00	
Level of ethanol consumption during a typical occasion in the past 30 days				1.00	
Low or medium	17.0	1.00		1.99 (1.05-3.76)	0.035
High or very high	28.7	1.96 (1.14-3.39)	0.02		
Ever used drugs				2.00 (1.00-4.01)	0.05
Yes	35.6	2.92 (1.66-5.12)	< 0.001	1.00	
No	15.9	1.00			
HIV-infected				2.44 (0.78-7.63)	0.124
Yes	50.0	4.03 (1.53-10.62)	0.005	1.00	
No	19.9	1.00			

[a] OR, odds ratio; CI, confidence interval. [b] Obtained from univariate logistic regression analyses. [c] Obtained from multivariate logistic regression analysis adjusting for potential confounding variables listed in the table.

sex after drinking. According to the multivariate logistic regression analysis, the demographic characteristics of ethnicity, education level, marital status, smoking status, frequency of drinking in the past month, and HIV infection status were all not significantly associated with sex after drinking (Table 4).

4. Discussion

To the extent known, this is the first community-based study examining alcohol use, sex after alcohol use, and their association with HIV infection among ethnic minorities in China. Findings revealed a high prevalence of lifetime alcohol use (90.3%), alcohol use in the past 30 days (89.8% among ever drinkers), and lifetime sexual activity after drinking (21.6%) among male residents of villages with mixed ethnic populations in rural Yunnan, China. The prevalence of alcohol use noted in this study is similar to that in males who are ethnic Li in Hainan Province (*26*) but is much higher than that in the general male Han population in China (though regional and local variations do exist). Hao *et al*. found that 63.8% of Chinese men had at least one drink in the past three months (*27*). The prevalence of alcohol use over 12 months among men was 54.4% in Beijing, the national capital (*28*), but nearly 90% in Wuhan, the capital of Hubei Province (*29*). The prevalence of alcohol use noted in the current study is higher than that in a study by Nehl *et al*. of a sample of MSM in Shanghai, 90% of whom reported ever drinking and 73.5% of whom reported moderate or high levels of alcohol consumption in the past three months (*15*). Furthermore, high levels of alcohol consumption in the past 30 days were noted in 31.9% of the current study participants, and this figure is higher than the prevalence of episodic heavy drinking over 12 months (24.6%) among men in Beijing (*28*). Based on the number of drinks per day in the past three months, Nehl *et al*. categorized 10.6% of MSM participants as heavy drinkers (two or more drinks per day) (*15*). Thus, the present study provides important information on the prevalence of alcohol use in a community with ethnic minorities that are severely affected by alcohol use, drug use, and HIV and that yet rarely receive public health attention.

Compared to other ethnic groups, Jingpo participants described significantly higher levels of heavy alcohol consumption. Li *et al*. argue that ethnic minorities in China (including Jingpo in Yunnan province) have a strong tradition of drinking, especially

during specific festivals, and tend to exhibit a higher prevalence of alcohol abuse and disorders than the Han majority (*30*).

In contrast to other studies in China (*15,27,31*), the present study indicates that homemade baijiu, rather than beer, is the most commonly consumed drink among ethnic communities in rural Yunnan. Such homemade baijiu has a very high ethanol content (usually over 40%) and is likely to be associated with an increased risk of harm due to unknown and potentially dangerous impurities or contaminants in these beverages (*4*). In fact, 14.5% of current drinkers in the current study reported having visited a clinic for an alcohol-related problem in the past year. These findings, along with the high percentages of daily drinking (44.5%) and morning drinking (13.5%) found among participants (especially among Jingpo participants), suggest that alcohol consumption could be a major social and public health problem, particularly for ethnic Jingpo males in rural Yunnan.

The global literature, including the limited number of studies in China, suggests that alcohol use and abuse are positively associated with a number of risky sexual behaviors and outcomes such as unwanted pregnancy and sexually transmitted infections (STIs) including HIV and syphilis (*10-18,32-35*). However, these studies often examine global instead of temporal (*i.e.*, sex after drinking) associations. In the present study, the lifetime prevalence of sexual activity after drinking was 21.6% among participants, and this figure was significantly higher among those who started drinking at an early age, who were heavy drinkers, and who were drug users. Moreover, condom use during sex after drinking was very rare, which is probably due to impaired judgment after alcohol use (*36,37*).

This study has several limitations. First, this was a cross-sectional study, so causal inferences cannot be made. Second, alcohol use and sexual behaviors are sensitive personal topics, so any self-reported measures are subject to recall bias, including deliberate concealment. Finally, participants were only tested for HIV infection but not for other STIs such as syphilis and herpes simplex virus-2 that have been found to be prevalent among Chinese men (*38*). Future studies should be extended to examine these STIs as outcomes of sex after alcohol use.

Nonetheless, findings from this study have important implications for future harm reduction programs targeting alcohol use and abuse in rural Yunnan, and particularly among ethnic Jingpo males. According to a 2003 Cochrane review on alcohol use in China (*39*), many high school students, especially boys, experience early alcohol initiation. Specifically, 55.7% of ever drinkers started drinking at the age of 18 or younger, suggesting that there is an urgent need to implement health education and intervention programs to prevent early use and misuse of alcohol among adolescents in rural Yunnan. Second, use of substances such as tobacco, alcohol, and illicit drugs has become a severe social and public health challenge that requires tremendous and integrated efforts in terms of research and control. Finally, given that Yunnan is one of China's HIV epicenters, the high prevalence of sex after alcohol use and the low rate of condom use during sex underscore the importance of enhanced condom promotion programs and empowerment programs for women to encourage condom use.

Acknowledgements

The authors wish to thank two village doctors, Drs. Zuluan Yin and Yongcui Yin, for their invaluable assistance in recruiting subjects and Mr. Brian Smith for editorial assistance. This study was supported by the National Natural Science Foundation of China (grant no. 81072345; PI: He). Preparation of this manuscript was also partially supported by the NIH/NICHD (grant No. R01HD056956; PI: Wong) and the Emory Center for AIDS Research (P30 AI050409; PI: Wong).

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(*Received October 24; Accepted December 12, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.6.296

A cross-sectional study of sputum handling by and supervision of patients with pulmonary tuberculosis treated at home in China

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Summary **Disposal of sputum from patients with pulmonary tuberculosis (TB) who are treated at home is an important aspect of preventing the spread of TB. However, few studies have examined disposal of sputum by patients with TB who are treated at home. Patients with pulmonary TB who are treated at home were surveyed regarding sputum handling and supervision. A cross-sectional survey of a representative sample of patients with pulmonary TB who are treated at home was conducted in Shandong Province. Participants were individuals with TB who had been registered with a local agency responsible for TB control. Participants completed a questionnaire with both qualitative and quantitative questions. How sputum was handled was determined and factors associated with sputum disposal were analyzed using a non-parametric test, logistic regression, and content analysis. Responses were received from 720 participants. Patients expectorated sputum 4.56 ± 10.367 times a day, and 68.6% of patients responded that they correctly disposed of their sputum. Supervision as part of TB control focused on the efforts of health agencies and paid little attention to waste management by patients. A non-parametric test showed that sputum disposal was significantly associated with gender, age, education, sputum smear results, attitudes toward waste management, and attitudes toward supervision (all $p < 0.05$). Logistic regression analysis showed that gender (OR = 0.482, 95% CI: 0.329-0.704), sputum smear results (OR = 1.300, 95% CI: 1.037-1.629), and level of education (OR = 0.685, 95% CI: 0.528-0.889) were associated with receipt of TB health education (all $p < 0.05$). Sputum handling by and supervision of patients with pulmonary TB who are treated at home is severely wanting. From a policy perspective, special attention should be given to the definition, details, and methods of supervision of waste management by patients with TB to give them relevant health education and enhance their willingness to be supervised. A financial incentive should be provided to health workers supervising management of TB-related waste.**

Keywords: Waste management, sputum, tuberculosis, supervision, cross-sectional survey

1. Introduction

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis*. It typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). TB can be transmitted in several ways, including *via* the respiratory tract and *via* the gastrointestinal tract. Several unusual methods of transmission have recently been reported, *e.g.* infections as a result of the use of contaminated equipment that was poorly cleaned (*1-3*). However, TB infection usually occurs in the lungs, with infection *via* the respiratory tract accounting for 95.93% of TB transmission (*4*). The main route of TB

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infection is *via* the respiratory tract, which includes droplet infection and airborne infection. Given this fact, incorrect waste management by patients with TB can result in aerosolized bacteria that will infect others who come into contact with the bacteria. Poor management of TB-related waste has been reported as a risk factor for increasing susceptibility to active TB infection (*5-8*). Therefore, studies have begun to focus on ways to appropriately manage waste from health care facilities as part of TB control (*9,10*). Because of the key role patients play in managing TB-related waste, supervision of waste management by patients with TB is also an important component of TB control. Therefore, waste management by patients with TB has been supervised in China since 1992 (*11*).

TB is a chronic disease with a long course of treatment. Because of social, financial, and family factors, most patients are treated at home (*12,13*). Thus, there is a need to examine waste management by patients with TB who are treated at home (PTBTHs). According to the existing data, TB control policies in China encompass supervision. However, current waste treatment by and supervision of PTBTHs has seldom been studied. This study defined waste management by patients with TB as how patients disposed of sputum, sputum cups, everyday items (clothes, bedding, tableware, and rags), and medical waste (respirators, handkerchieves, tissue paper, pharmaceutical packaging, infusion apparatuses, and syringes). Therefore, supervision of waste management should focus on the items listed. This study sought to explore current sputum disposal and supervision of that disposal, both of which are crucial to waste management by PTBTHs.

2. Materials and Methods

2.1. *Materials*

Multi-stage random sampling was used to select Weihai, Ji'nan, and Linyi as survey sites based on their level of economic development and geographical location (eastern, central, western regions) in Shandong Province. In each city, 2 counties were selected, and in each county 6 towns were selected at random. This study was conducted from August to September 2012. The three districts represent three levels of economic development in Shandong Province (Linyi: slow, Ji'nan: moderate, and Weihai: rapid).

In 2011, Linyi had a per capita GDP of 20,869 RMB (approximately 3,161 US dollars) and a population of 10,150,411, Ji'nan had a per capita GDP of 58,533 RMB (approximately 8,869 US dollars) and a population of 5,900,768, and Weihai had a per capita GDP of 69,858 RMB (approximately 10,585 US dollars) and a population of 2,483,889 (*13*). The current survey focused on PTBTHs and 720 PTBTHs were interviewed. The Ethics Committee of Shandong University approved this study. The study was conducted after obtaining informed consent from all participants.

2.2. *Methods*

In-depth interviews were conducted with patients to ascertain how they disposed of their sputum. A semi-structured questionnaire was designed by the Institute of Social Medicine and Health Services Management, School of Public Health, Shandong University, China. It included general status, economic indicators, TB status, changes in living habits, awareness of and attitudes towards waste management, social activities, and cell phone and Internet use.

Quantitative data were analyzed with SPSS version 13.0 statistical software. Descriptive analysis and a non-parametric test were performed on quantitative data.

Content analysis was used to analyze qualitative data. All aspects influencing sputum handling at home that patients brought up in interviews were listed, and then a card was made for each aspect mentioned. Similar aspects were grouped together, and then the groups were arranged into a relationship model in a logical order.

The Ethical Research Committee of Shandong University approved this study and informed consent was obtained from each patient. All participants were informed about the study procedures and the survey was conducted after informed consent was obtained.

3. Results

3.1. *General information on TB control in China*

TB is the leading cause of death from a curable infectious disease. TB Impact Measurement revealed an estimated 8.7 million incident cases of TB (range, 8.3 million-9.0 million) globally, equivalent to 125 cases per 100,000 population in 2011 (*4*). The five countries with the largest number of incident cases in 2011 were India (2.0 million-2.5 million), China (0.9 million-1.1 million), South Africa (0.4 million-0.6 million), Indonesia (0.4 million-0.5 million), and Pakistan (0.3 million-0.5 million). China alone accounted for 12% of global cases. There were an estimated 899,669 new cases of TB in China, 56% of which had positive sputum smears (the most infectious cases).

Since the Directly Observed Treatment Short Course strategy (DOTS) was implemented in China in 1992, China has made great progress in TB prevention and treatment over the last few decades. In 2006, the DOTS coverage rate was 100% at the county level, the detection rate for new smear-positive TB cases was 79%, and the cure rate was 92% (*11*). During implementation of the DOTS program, supervision plays a very important role in informing community members of TB symptoms and transmission to help

prevent its spread. Supervision has been shown to improve sputum disposal by providing patients with the information necessary to understand the importance of sputum disposal (*14-18*).

However, the supervision of patients with TB is still inadequate. Supervision as part of TB control in China emphasizes efforts by health agencies, such as specimen collection, analysis of TB information, and monitoring of TB epidemics. Health education is focused more on identifying TB cases and ensuring that medication is taken in the right combination and appropriate dosage while observing patients taking TB medications (*19,20*). Therefore, waste management by patients with TB has received insufficient attention in China.

3.2. *General information on PTBTHs*

Among the sample of 720 patients with TB in Shandong Province, those treated within a month expectorated sputum 4.56 ± 10.367 times a day. Asked how they disposed of their sputum before diagnosis, 384 patients spit casually, 89 patients used a sputum cup, none of the patients buried their sputum, 81 patients used a handkerchief to collect their sputum, 51 patients spit in a designated location, and 117 patients said they used other methods to dispose of their sputum or that they had no sputum (Figure 1). Asked about how they disposed of their sputum in the first month of treatment, 130 patients spit casually, 124 patients used a sputum cup, 37 patients chose to bury their sputum, 126 patients used a handkerchief to collect their sputum, 100 patients spit in a designated location, and 245 patients said they used other methods to dispose of their sputum or that they had no sputum (Figure 2). These findings indicate that the number of patients who chose to spit casually decreased significantly and the number of patients who appropriately disposed of their sputum increased.

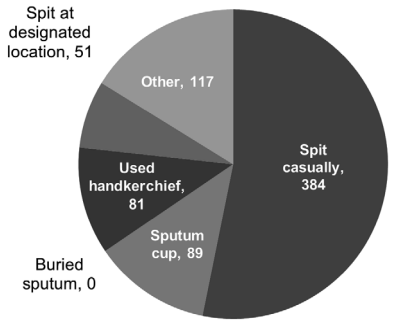

Figure 1. Handling of sputum before diagnosis.

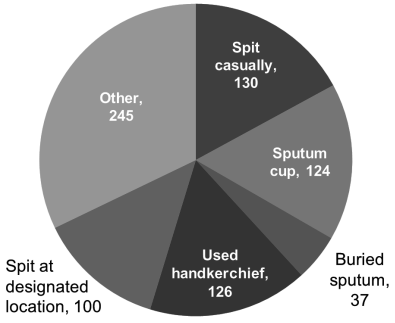

Figure 2. Handling of sputum during the first month of treatment.

Differences in the characteristics of patients with TB who correctly disposed of sputum (DS+ patients) and those who incorrectly disposed of sputum (DS− patients) are shown in Table 1. Differences among age groups were noted ($p < 0.05$): 80.5% of patients 10-25 years of age were DS+ patients, 80.0% of those 26-40 years of age were DS+ patients, 72.5% of those 41-55 years of age were DS+ patients, 60.9% of those 56-70 years of age were DS+ patients, and 67.0% of those ≥ 70 years of age were DS+ patients.

The ratio of male to female patients was 2.36:1 (506/214). Gender differences were noted since 64.2% of men and 79.0% of women disposed of sputum correctly. By level of education, 64.5% of patients with no more than a primary school education disposed of sputum correctly, 70.9% with a high school education did so, and 82.9% with a technical secondary school or higher education did so. The difference in correct disposal of sputum by patients with different levels of education was statistically significant ($p < 0.05$). With regard to treatment outcomes, 50.3% of patients had been cured and 47.4% were undergoing treatment. Of 362 cured patients, 68.8% responded that they disposed of sputum correctly and 68.3% of the patients undergoing treatment did so. With regard to sputum smear results, 16.1% of the patients were had positive smears, 33.6% had negative smears, and 50.3% had smear results that were not known. Of 116 smear-positive patients, 74.1% responded that they disposed of sputum correctly as did 74.0% of smear-negative patients and 63.3% of patients with smear results that were not known. A statistically significant difference was noted in terms of sputum smear results ($p < 0.05$).

Economics played a role since 44.6% of patients had a salary of 0-9,999 RMB while 55.4% had a salary of ≥ 10,000 RMB group. However, the proportion of patients who correctly disposed of sputum did not differ statistically between income levels ($p = 0.135$).

Of the patients, 59.9% responded that they cared about waste management, 34.3% thought it unnecessary, 5.8% had no feelings either way. The difference in sputum disposal in terms of attitudes toward waste management was statistically significant ($p < 0.05$): 72.2% of the patients who considered waste management necessary correctly disposed of sputum, 63.6% of the patients who considered it unnecessary did so, and 61.9% of the patients with no feelings

Table 1. Differences in characteristics of patients with TB who dispose of their sputum correctly (DS+) and incorrectly (DS−)

Item	Dispose of sputum correctly		Dispose of sputum incorrectly		Statistics	*p*
	n	(%)	*n*	(%)		
Gender					15.178	< 0.001
Male	325	64.2	181	35.8		
Female	169	79.0	45	21.0		
Age (years)					19.523	0.001
10-25	66	80.5	16	19.5		
26-40	64	80.0	16	20.0		
41-55	108	72.5	41	27.5		
56-70	179	60.9	115	39.1		
70-	77	67.0	38	33.0		
Education					10.238	0.006
Primary school or less	251	64.5	138	35.5		
High school	185	70.9	76	29.1		
Technical secondary school or higher	58	82.9	12	17.1		
Yearly salary (RMB)					2.229	0.135
0-9,999	211	65.7	110	34.3		
10,000-	283	70.9	116	29.1		
Treatment outcome					0.049	0.976
Cured	249	68.8	113	31.2		
Treatment underway	233	68.3	108	31.7		
Other	12	70.6	5	29.4		
Sputum smear results					9.682	0.008
Positive	86	74.1	30	25.9		
Negative	179	74.0	63	26.0		
Not known	229	63.3	133	36.7		
Attitudes toward waste management					6.317	0.042
Necessary	311	72.2	120	27.8		
Unnecessary	157	63.6	90	36.4		
No feeling either way	26	61.9	16	38.1		
Attitudes toward supervision					11.130	0.004
Necessary	325	72.9	121	27.1		
Unnecessary	117	59.7	79	40.3		
No feeling either way	52	66.7	26	33.3		
Received health education					3.073	0.080
Yes	343	70.7	142	29.3		
No	151	64.3	84	35.7		
Supervised					2.781	0.095
Yes	164	72.9	61	27.1		
No	330	66.7	165	33.3		

either way did so. With regard to supervision, 62.0% responded that supervision was necessary, 27.2% thought it unnecessary, and 10.8% had no feelings either way. A statistically significant ($p < 0.05$) difference was noted in terms of attitudes since 72.9% of patients who considered supervision necessary disposed of sputum correctly, 59.7% of patients who considered it unnecessary did so, and 66.7% of patients with no feelings either way did so.

Asked about receiving health education, 67.4% of patients received health education and 32.6% did not. Of 485 patients who received health education, 70.7% responded that they correctly disposed of sputum while 64.3% of patients who received no such education responded that they did so. Asked whether they were supervised, 31.3% of the patients were supervised while 68.7% were not. Of 225 patients who were supervised, 72.9% responded that they disposed of sputum correctly while 66.7% of those who were not supervised did so. However, the proportion of patients who correctly disposed of sputum did not statistically differ for those who received health education ($p = 0.080$) or who were supervised ($p = 0.095$).

Supervision details such as supervisors, approaches, scope, and frequency were also ascertained. Among the sample of 720 PTBTHs, 225 patients (31.2%) confirmed that they had been supervised and 495 patients (68.8%) said they had not been supervised. Among the 225 patients who were supervised, 27.6% said that family members supervised waste management, 13.8% said that a rural doctor did so, and 17.8% said that a doctor at a township hospital did so. Of the 225 patients, 65.8% confirmed that staff of the county-level agency responsible for TB control supervised their management of TB-related waste. Asked about the approach to supervision, 6.2% of patients were supervised in writing, 30.7% were supervised face-to-face, and 18.2% were supervised by phone; 60.4% of patients were supervised during treatment. Regarding the scope of supervision, 78.2% of patients were instructed in sputum disposal, 43.1% were instructed in use of items such as a handkerchief or rag, 34.2% were instructed in use of a sputum cup, 25.3% were instructed in handling of medication packaging, 22.7% were instructed in handling of syringes and transfusion systems, 28.9% were instructed in handling of used clothing, bedding, and other everyday items, 33.3% were instructed in handling of used tableware, 32.4% were instructed in

Table 2. Logistic regression analysis of influencing variables

Risk factor	B	S.E.	OR	95% CI for OR Lower / Upper	*p*
Sputum smear results	0.262	0.115	1.300	1.037 / 1.629	0.023
Gender	– 0.730	0.194	0.482	0.329 / 0.704	< 0.001
Level of education	– 0.378	0.133	0.685	0.528 / 0.889	0.004

handling of used tissues, and 30.7% were instructed in handling used respirator. Supervision took place at a frequency of 4.70 ± 7.415 times.

A logistic regression model was used to identify factors influencing sputum disposal by PTBTHs. Table 2 shows the influencing factors identified by logistic regression. A finding of significance in an omnibus test of model coefficients ($p < 0.0001$) indicated that data were adequate fitted to the model.

Three factors were associated with sputum disposal by PTBTHs: sputum smear results, gender, and level of education. PTBTHs who were unaware of their sputum smear results were less likely to dispose of sputum correctly compared to those who were aware of their results (OR = 1.300, 95% CI: 1.037-1.629). Female patients were more likely to dispose of sputum correctly than were male patients (OR = 0.482, 95% CI: 0.329-0.704). PTBTHs who were more educated were more likely to dispose of sputum correctly (OR = 0.685, 95% CI: 0.528-0.889).

3.3. *Results of qualitative research*

In addition to quantitative data, this study provided qualitative information regarding patients' ideas of and views on sputum disposal, health education, and supervision.

The survey revealed that waste management by patients with TB is still lacking. As an example, a TB treatment room accommodating four patients had only one window and no ventilation. There were plastic sputum cups with a plastic bag inside that were supposedly disinfected. Staff were asked how they sterilized the sputum cup but they answered that they simply threw away the plastic bag with sputum inside. Staff were asked about the health education they provided and they said that health education was provided every time patients visited; they also mentioned other forms of health education such as bulletin boards and noncommercial ads. Asked about supervision, staff responded that supervision was inadequate compared to health education. Recent supervision has focused more on administrative aspects such as management of funds, detection, management of medication. Waste management by patients is still not adequately supervised.

Staff cited the importance of health education during sputum disposal. They felt that patients with adequate health education would better understand how to correctly dispose of sputum and thus try to do so. Several such views were expressed:

'The expert said that correct disposal of sputum helps to control TB... spitting casually is not good for yourself or others, so I never spit casually. I always carry a plastic bag (to spit in) when I go out...'

'During my treatment, the doctor always told to me how I should dispose of sputum and what might happen if I spit casually... of course, I will do what's right for myself and others...'

Some patients mentioned staff attitudes towards supervision. They described being reminded by others to act appropriately whereas patients who received no such attention expressed less concern:

'The doctor talks to me every time he sees me on the street and asks me how I feel and how my treatment is going... the TB supervisor asks me if I am taking my medicine and what else I'm doing... I will do what they told me and get cured as soon as possible (because of this kind of care)...'

'Since nobody cares about how I feel or how I am, why should I care about my health behavior? After all, I'm the one who's sick.'

However, current supervision has drawbacks. Patients who were supervised were asked about their supervision. They said that supervision was flawed and not standardized or consistent:

'...the doctor asked about my treatment all the time... he always mentioned the medicine... he didn't seem to care much about how I disposed of my sputum...'

'Of course, no one does that job (supervision) regularly. Sometimes they said something about it (supervision), and sometimes they didn't.'

Some patients said that they do not want to be supervised or receive health education because they are afraid of being discriminated against by others who would realize that they had TB:

'I know this disease is contagious, but I don't want to be supervised or receive health education... other people will know that I have TB if I do, and I will be discriminated against...'

4. Discussion

This study found that 68.6% of the PTBTHs in Shandong Province responded that they correctly disposed of sputum. The rate of appropriate sputum disposal by PTBTHs in China is consistent with the high rate of DOTS coverage and other quality indicators

for TB treatment reported by the Ministry of Health of China.

This study noted statistically significant differences in sputum disposal by PTBTHs in terms of gender, age, level of education, sputum smear results, attitudes toward waste management, and supervision. This study identified three statistically significant influencing factors: sputum smear results, gender, and level of education.

Health education has improved but is still inadequate. Of the patients surveyed, 67.4% had received health education. However, many patients described a lack of concern during treatment and health education. The approach to health education was mostly face-to-face education during a visit. New approaches to health education must be explored.

This study found that the current supervision of PTBTHs is still inadequate despite the important role waste management by patients plays in TB control. Of patients, 61.9% were willing to be supervised and 31.3% were actually supervised. Although family members and agencies responsible for TB control help PTBTHs to manage their waste, rural doctors and doctors in township hospitals should enhance their supervision of waste management by PTBTHs. Methods of supervision are still limited. Being supervised face-to-face and being supervised during treatment were the predominant forms of supervision, and further attention should be directed toward more efficient and effective supervision *via* technology such as use of phone calls or the Internet. The scope of supervision also needs to be expanded. Used sputum cups, used tableware, everyday items, and used respirators can also spread TB (*21,22*). The handling of these items must be supervised to control TB. Medical staff and patients indicated that supervision is focused more on supervising administrative aspects and patient treatment. Specialized health workers in agencies responsible for TB control are expected to better supervise the population. That said, caution is required with regard to these specialists' supervision of waste management by PTBTHs. Health workers currently have no firm rules regarding how they should supervise waste management by patients with TB and what they should do when supervising those patients, so they do not know what to do or how to do it. For PTBTHs, failure to appreciate the importance of waste management and the fear of been discriminated against are major barriers to a willingness to be supervised. These aspects may have led to the current findings.

In order to improve sputum disposal by PTBTHs, the current study suggests that greater attention must be paid to the definition, details, and methods of supervision of waste management by patients with TB from a policy perspective. Supervision with specific aims is more effective, which may be explained by the fact that doctors who have received clear guidelines are better able to supervise management of TB-related waste (*23,24*). Defining the supervision of waste management by patients with TB results in health workers who are better able to effectively and efficiently supervise the management of TB-related waste.

This study found that another factor, the willingness of PTBTHs to be supervised, has a large impact on the actual receipt of health education. Therefore, this study suggests that providing PTBTHs with relevant health education and encouraging a willingness to be supervised can help improve their sputum disposal. This might be explained by the fact that patients who have received health education are better able to manage TB-related waste on their own (*25*). More relevant health education must be developed so that PTBTHs are better able to manage TB-related waste on their own.

This study also suggests that health workers should be provided a financial incentive to supervise the management of TB-related waste. Awareness of national financial incentives has been indispensable to the supervision of the management of TB-related waste. Health workers should be financially rewarded for supervising management of TB-related waste. Health workers need to be directly rewarded for their supervision of the management of TB-related waste. A previous study examined the importance of personal needs with a focus on economic rewards (*26*).

In summary, most PTBTHs responded that they correctly disposed of their sputum. In addition, the factors of gender, level of education, and sputum smear results were found to be associated with sputum disposal by PTBTHs. These findings will help to plan future strategies to encourage correct sputum disposal by PTBTHs as part of TB control and prevention.

Acknowledgements

This work was supported by a grant from the Department of Science & Technology of Shandong Province as a Science and Technology Development Project (project ID: 2012GSF11843). The authors also wish to thank the Departments of Health of Ji'nan, Weihai, and Linyi for their assistance.

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(*Received November 9, 2012; Revised December 15, 2012; Accepted December 19, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.6.303

Effects of two monoclonal antibodies, MLS128 against Tn-antigen and 1H7 against insulin-like growth factor-I receptor, on the growth of colon cancer cells

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Summary **MLS128 is an anti-carbohydrate monoclonal antibody (mAb) that binds three or two consecutive Tn-antigens. MLS128 bound 110-210 kDa glycoproteins (GPs) and inhibited the growth of LS180 and HT29 colon and MCF-7 breast cancer cells. One possible mechanism of MLS128's inhibition of growth may be *via* insulin-like growth factor-I receptor (IGF-IR) down-regulation (*Morita et al. BioSci Trends. 2009; 3:32-37*). The current study examined the role of IGF-IR signaling in the growth of colon cancer cells and its possible interaction with MLS128-induced inhibition of cell growth in LS180, LS174T, and HT29 human colon cancer cells treated with MLS128 or anti-IGF-IR 1H7. Both MLS128 and 1H7 treatment significantly inhibited the growth of colon cancer cells. All three colon cancer cell lines expressed IGF-IR. Their growth was in part IGF-I dependent, but inhibition by MLS128 was independent of IGF-IR signaling. All of the colon cancer cell lines expressed an 110 kDa GP for MLS128 binding, but MCF-7 cells expressed MLS128-detectable bands with higher molecular masses. 1H7 treatments caused down-regulation of IGF-IR but did not affect 110 kDa GP levels. MLS128 treatments resulted in partial disappearance of the 110 kDa band but did not affect IGF-IR levels. Western blotting analyses of colon and breast cancer cell lysates revealed that colon and breast cancer cells differed significantly in patterns of expression of growth-related molecules while colon cancer cells were similar but distinctive. In conclusion, MLS128 inhibited the growth of colon cancer cells by binding to the 110 kDa GP receptor. Inhibition of growth by MLS128 did not appear to affect IGF-IR signaling and instead only affected other growth signaling pathways.**

Keywords: Colon cancer, mechanisms of growth inhibition, anti-Tn antigen, anti-IGF-I receptor

1. Introduction

MLS128 is a Tn-antigen specific monoclonal antibody (mAb) (*1*) that binds carbohydrate epitopes consisting of three or two consecutive Tn-antigens (*2-4*). A previous study showed that MLS128 bound 110-210 kDa glycoproteins on cell membranes and inhibited the growth of LS180 and HT29 colon cancer cells as well as MCF-7 breast cancer cells (*5*). One possible mechanism of the inhibition of growth by MLS128 may be *via* insulin-like growth factor-I receptor (IGF-IR) down-regulation (*5*). Thus, the current study focused on the role of IGF-IR signaling in the growth of colon cancer cells and its possible interaction with MLS128-induced inhibition of cancer cell growth.

IGF-IR signaling is known to play an important role in proliferation, anti-apoptosis, and differentiation (*6,7*). IGF-I and -II bind to IGF-IR with a high affinity and thus activate IGF-IR tyrosine-kinase, which in turn stimulates downstream signaling cascades. Increased IGF-IR signaling has been reported to contribute to cancer cell growth and development (*8,9*). IGF-IR is thus an important target for cancer treatment (*10*). In

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order to develop cancer therapeutics, one of the current authors (Y. F.-Y.) previously constructed a single-chain variable fragment (scFv) from the variable domains of 1H7 mAb (*11*), produced it as a chimeric scFv-Fc consisting of anti-IGF-IR mouse scFv and human IgG1 Fc domain, and showed that it inhibited MCF-7 tumor growth (*12*). Inhibition of MCF-7 and T61 breast tumor growth by the scFv-Fc *in vivo* was caused by down-regulation of IGF-IR (*13,14*). Subsequent research suggested that down-regulation due to internalization and degradation is a major mechanism by which anti-IGF-IR antibodies inhibit cell growth (*15-17*).

One cannot reasonably assume, however, that the growth of colon cancer cells depends solely on IGF-IR signaling. In fact, the current study revealed that addition of anti-IGF-IR mAb 1H7 significantly inhibited cell growth of all colon cancer cell lines studied but that this inhibition was only approximately 60%. Individual cancer cells are likely to be equipped with diverse growth signaling pathways, and one or two particular signaling pathways may play a major role in growth of a particular cancer cell line. Expression of several possible growth-related surface molecules has thus been measured in three colon cancer cell lines to ascertain different mechanisms that might play a role in the growth of colon cancer cells. Western blotting analyses of colon and breast cancer cell lysates revealed that colon and breast cancer cells differ significantly with regard to their patterns of expression of growth-related molecules. Although three colon cancer cell lines expressed molecules in similar patterns, all three were distinctive. In summary, this study demonstrated that 1H7 and MLS128 inhibited the growth of colon cancer cells. The 110 kDa GP has been identified as the MLS128 binding receptor in colon cancer cells. Colon cancer cells were in part IGF-IR signaling-dependent. Inhibition of growth by MLS128 does not, however, apparently depend on IGF-IR signaling, suggesting that MLS128 likely inhibits the growth of colon cancer cells by affecting other growth signaling pathways.

2. Materials and Methods

2.1. *Materials*

Production and characterization of MLS128 were previously described (*1-4*). LS180, LS174T, and HT-29 human colon adenocarcinoma cells as well as MCF-7 human breast carcinoma cells were obtained from the American Tissue Type Culture Collection. Rabbit anti-IGF-IRβ, anti-EGFR mAb, rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204), rabbit anti-phospho-Akt (ser473) (193H12), rabbit anti-p44/42 MAPK, rabbit anti-CD44, anti-phosphotyrosine mAb, and rabbit anti-β actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-c-Met and anti-E-cadherin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit or -mouse secondary antibody labeled with biotin was from Kirkegaard & Perry Lab. (Gaithersburg, MD, USA). IGF-I was obtained from Sigma-Aldrich Biotechnology (St. Louis, MO, USA). Cell culture media (DMEM and McCoy's 5A) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. *Cell culture*

LS180 and LS174T cells were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 4.5 mg/mL D-glucose and 110 μg/mL pyruvic acid. HT29 cells were cultured in McCoy's 5A containing 10% FBS. MCF-7 cells were cultured in IMEM (GIBCO, Gland Island, NY, USA) containing 5% FBS and 11.25 nM insulin. All culture media included 1% Penicillin-Streptomycin solution (Sigma-Aldrich).

2.3. *Effects of 1H7 or MLS128 on the growth of colon cancer cells*

Cells (1×10^4) were plated in wells of a 96-well plate and cultured in 100 μL of respective media containing 10% FBS for 24 h. Attached cells were then washed twice with PBS and cultured in 100 μL of media containing 1% FBS in the presence or absence of MLS128 (25 μg/mL) or 1H7 (0.36 μg/mL). After culturing for 72 h, cells were collected from each well by treatment with Trypsin-EDTA (Sigma-Aldrich) for 10 min followed by centrifugation at 200 × g for 10 min. Cell pellets were suspended in 0.04% Trypan blue (Sigma-Aldrich). Live cell numbers were counted using a hemacytometer. Quadruple wells were prepared for each data point.

2.4. *Effects of IGF on the growth of colon cancer cells*

To measure the effects of IGF-I on the growth of colon cancer cells, cells (5×10^3) were plated in wells of a 96-well plate and cultured in 100 μL of respective medium containing 10% FBS for 24 h. Attached cells were washed twice with PBS and serum-deprived for 24 h in regular growth media containing 0.1 % BSA instead of FBS (SFM). Media were replaced with SFM containing 0, 0.76, or 76 ng/mL of IGF-I. After culturing for 24, 48, and 72 h, cell growth was determined using a CCK-8 cell counting kit (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. Absorbance at 450 nm was measured with a plate reader (Bio-Rad, Hercules, CA, USA). Quadruple wells were prepared for each data point.

2.5. *Western blotting analyses of various growth-related proteins in colon and breast cancer cells*

LS180, LS174T, and HT-29 colon cancer cells as well

as MCF-7 breast cancer cells were cultured in their respective media. Cells were collected by scraping, followed by centrifugation at 200 × g for 5 min, and then solubilized in 50 mM Tris-HCl buffer, pH 7.4, containing 1% NP40, 2 mM EDTA, 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM PMSF and protease inhibitors (lysis buffer) on ice for 15 min. Supernatants were obtained from solubilized cells by centrifugation at 17,000 × g for 10 min. Protein concentrations were measured using the Bradford method. The solubilized proteins (5 and 25 μg) from each cell line were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membrane was blocked with 3% BSA in 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1% Tween 20 (TBST) for 1 h at room temperature. Western blotting was carried out with anti-IGF-IR, MLS128, anti-EGFR, anti-c-Met, anti-E-cadherin, and anti-CD44 antibodies as primary antibodies. Bound primary antibodies were then detected with biotin-labeled secondary antibodies using the Vectastain ABC-Amp kit and an alkaline phosphatase kit (Vector Laboratories, Inc. Burlingame, CA, USA). Alternatively, bound primary antibodies were detected with HRP-conjugated secondary antibodies and color development using Ez West blue (ATTO Co., Tokyo, Japan). The blots were analyzed using NIH Image 1.63 Analysis system (Research Service Branch, the National Institute of Mental Health).

2.6. *Effects of 1H7 or MLS128 treatment on IGF-IR and 110 kDa GP levels in colon cancer cell lines according to Western blotting analyses*

LS180, LS174T, and HT29 colon cancer cells (8×10^5) were cultured in respective media containing 10% FBS for 24 h in wells of 6-well plates. Cells were then cultured in the respective media containing 1% FBS in the presence or absence of MLS128 (25 μg/mL) or 1H7 (0.36 μg/mL). After culturing for 24, 48, and 72 h, cells were collected and solubilized in 50 μL of the lysis buffer as described above. The solubilized proteins (2 μg per lane) were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 3% BSA in TBST for 1 h at room temperature. After incubation with primary antibodies against 110 kDa GP (MLS128), IGF-IR β subunit, and β-actin for 16 h, bound primary antibodies were detected as described above.

2.7. *Effects of MLS128 on IGF-IR signaling in colon cancer cells*

Cells (8×10^5) were plated in wells of 6-well plate and cultured in respective medium containing 10% FBS for 24 h. Cells were then cultured in SFM containing 0.1% BSA or 25 μg/mL of MLS128. Fifty ng/mL of IGF-I was added to MLS128-treated and non-treated cells. After incubation for 5 or 10 min, cells were collected by centrifugation and subjected to solubilization in 50 μL of the lysis buffer for 15 min, followed by centrifugation at 17,000 × g for 10 min. Recovered supernatants containing solubilized proteins were assayed for protein concentrations. The solubilized proteins (5 μg per lane) were separated by SDS-PAGE and subjected to Western blotting as described above. Primary antibodies against p-Tyr, p-MAPK, and p-Akt were used for detection of IRS-1, MAPK, and Akt phosphorylation, respectively. After incubation at 4°C overnight, bound primary antibodies were detected using biotin-conjugated secondary antibodies and the Vectastain ABC-Amp kit (Vector Laboratories, Inc. Burlingame, CA, USA).

2.8. *Statistical analyses*

Levels of cell growth and Western blotted protein bands were expressed as means ± S.E. from 3 or more experiments. An unpaired Student's *t*-test was used to compare the growth or intensity of the bands in two groups of experiments performed in the absence and presence of mAb.

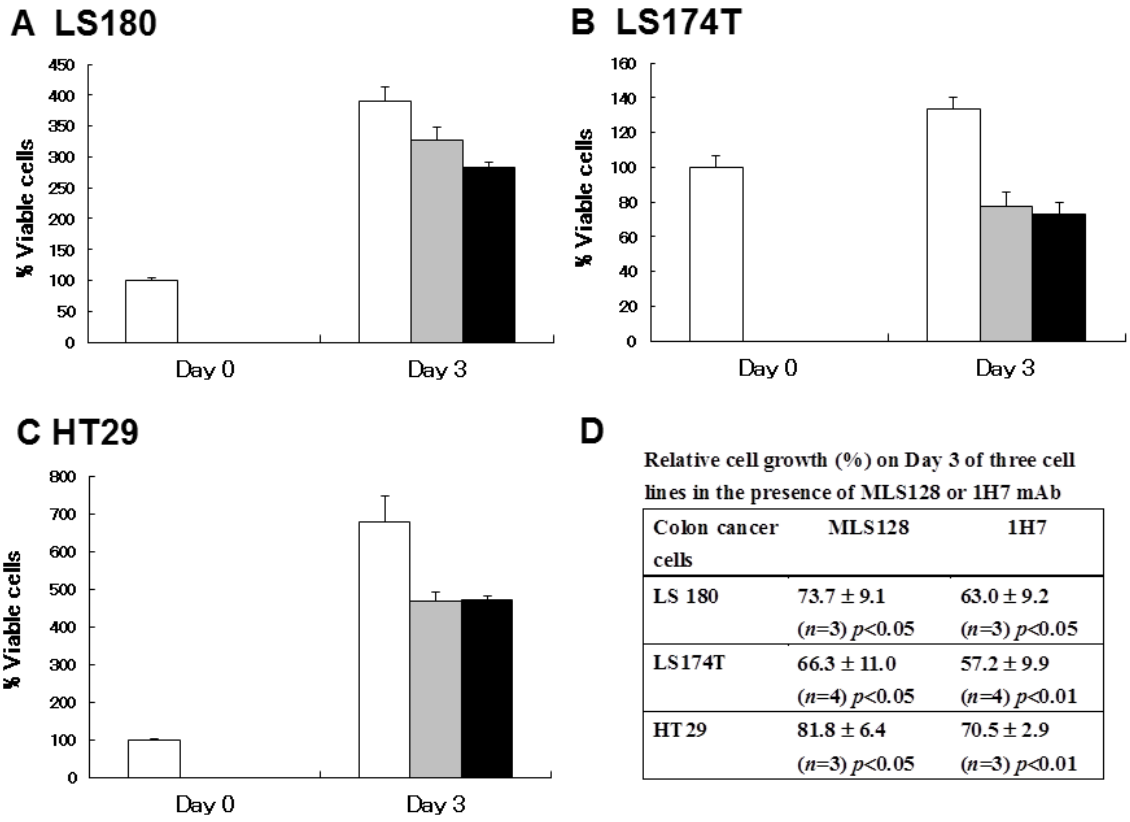
3. Results

3.1. *Addition of MLS128 or 1H7 inhibits growth of three colon cancer cell lines*

A previous study found that MLS128 inhibited the growth of LS180 and HT29 colon cancer cells as well as MCF-7 breast cancer cells (*5*). Thus, the current study compared the inhibition of growth of three colon cancer cell lines, including an additional LS174T colon cancer cell line. Typical results for inhibition of the growth of LS180, LS174T, and HT29 colon cancer cells after 3 days of mAb treatment are shown in Figure 1A, B, and C, respectively. The mean ± S.E. from 3 or 4 experiments with each cancer cell line is summarized in Figure 1D.

MLS128 treatment of LS180, LS174T, and HT29 colon cancer cells for 3 days significantly inhibited cell growth (Figure 1 A, B, and C, respectively). Results confirmed previous findings that MLS128 inhibited on inhibition the growth of LS180 and HT29 cells (*5*). In addition, inhibition of growth was also noted in the LS174T cell line, which is a variant of LS180 that had been maintained with using trypsin in accordance with a subculture protocol (*18*). As shown in Figure 1D, 3 days of MLS128 treatment inhibited the growth of LS180, LS174T, and HT29 colon cancer cells by an average of 26%, 34%, and 18%, respectively.

Anti-IGF-IR 1H7 significantly inhibited the growth of LS180, LS174T, and HT29 colon cancer cell lines by 37%, 43%, and 30%, respectively (Figure 1D). An interesting finding is that monolayer growth of colon cancer cells was significantly inhibited by 1H7. The



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Relative cell growth (%) on Day 3 of three cell lines in the presence of MLS128 or 1H7 mAb

Colon cancer cells	MLS128	1H7
LS 180	73.7 ± 9.1 (n=3) p<0.05	63.0 ± 9.2 (n=3) p<0.05
LS174T	66.3 ± 11.0 (n=4) p<0.05	57.2 ± 9.9 (n=4) p<0.01
HT29	81.8 ± 6.4 (n=3) p<0.05	70.5 ± 2.9 (n=3) p<0.01

Figure 1. Addition of MLS128 or 1H7 inhibits growth of three colon cancer cell lines. Inhibition of growth of three colon cancer cell lines by MLS128 (grey bars) along with anti-IGF-IR 1H7 (black bars) was compared to the control (white bars). Experiments were performed as described in the Methods. Typical results for growth of LS180, LS174T, and HT29 colon cancer cells after 3 days of mAb treatment are shown in **A**, **B**, and **C**, respectively. The relative growth (% control), average ± S.E. from 3 or 4 experiments for each cancer cell line, is summarized in **D**.

same was not true for monolayer MCF-7 cell growth, which was not significantly inhibited in the presence of 1H7 scFv-Fc (*13*). The situation differs with breast cancer: 1H7 scFv-Fc markedly inhibited anchorage-dependent MCF-7 cell growth *in vitro*, and MSF-7 and T61 breast cancer tumor growth *in vivo via* down-regulation of IGF-IR (*13,14*).

3.2. *Growth of colon cancer cells is IGF-dependent*

LS180 (A), LS174T (B), or HT29 (C) cell growth was observed under serum-free conditions in the absence or presence of IGF-I. The results shown in Figure 2 indicate that the growth of three colon cancer cell lines is in part IGF-I-dependent. These results are consistent with the finding that anti-IGF-IR 1H7 inhibited the growth of all three colon cancer cell lines (Figure 1).

3.3. *Effects of MLS128 on IGF-IR signaling in three colon cancer cell lines*

The effects of MLS128 on IGF-IR signaling were examined in three colon cancer cell lines. MLS128 did not affect the downstream signaling stimulated by IGF-I as determined by immunoblotting of p-Tyr, p-MAPK, and p-Akt (Figure 3). These results suggest that MLS128's inhibition of the growth of colon cancer cells is independent of IGF-IR signaling. While the effects of MLS128 on IGF-IR signaling were not obvious, MLS128 may have different effects on basal phosphorylation of MAPK and Akt, in that MLS128 inhibited MAPK and Akt phosphorylation in LS180 cells, by 0.67 ± 0.04 (n = 3)- and 0.61 ± 0.22 (n = 3)-fold, respectively, but it activated IRS-1 and MAPK phosphorylation in HT29 cells by 1.21 ± 0.08 (n = 3)- and 1.25 ± 0.05 (n = 3)-fold, respectively. These results indicate that MLS128 affects colon cancer cell signaling *via* as yet-unidentified signaling pathways other than the IGF-IR signaling pathway.

3.4. *Effects of 1H7 or MLS128 treatment on IGF-IR levels in colon cancer cell lines*

Since previous studies suggested that MLS128 inhibits LS180 cell growth *via* down-regulation of the IGF-IR (*5*), the amounts of IGF-IR in three colon cell lines after 1H7 or MLS128 treatment were determined by immunoblotting (IB). After treatment with MLS128 or

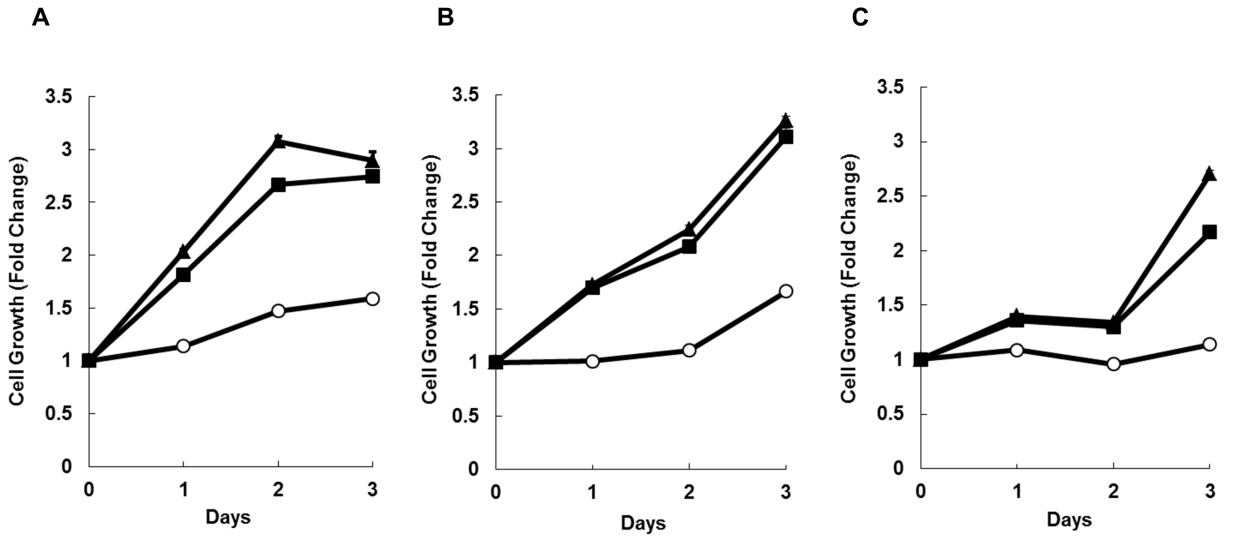

Figure 2. Growth of colon cancer cells is IGF-dependent. LS180, LS174T, or HT29 cell growth was observed under serum-free conditions in the absence (○) or presence of 0.76 (■) or 76 ng/mL (▲) of IGF-I for 3 days as described in the Methods. Typical results from 2-4 experiments are shown.

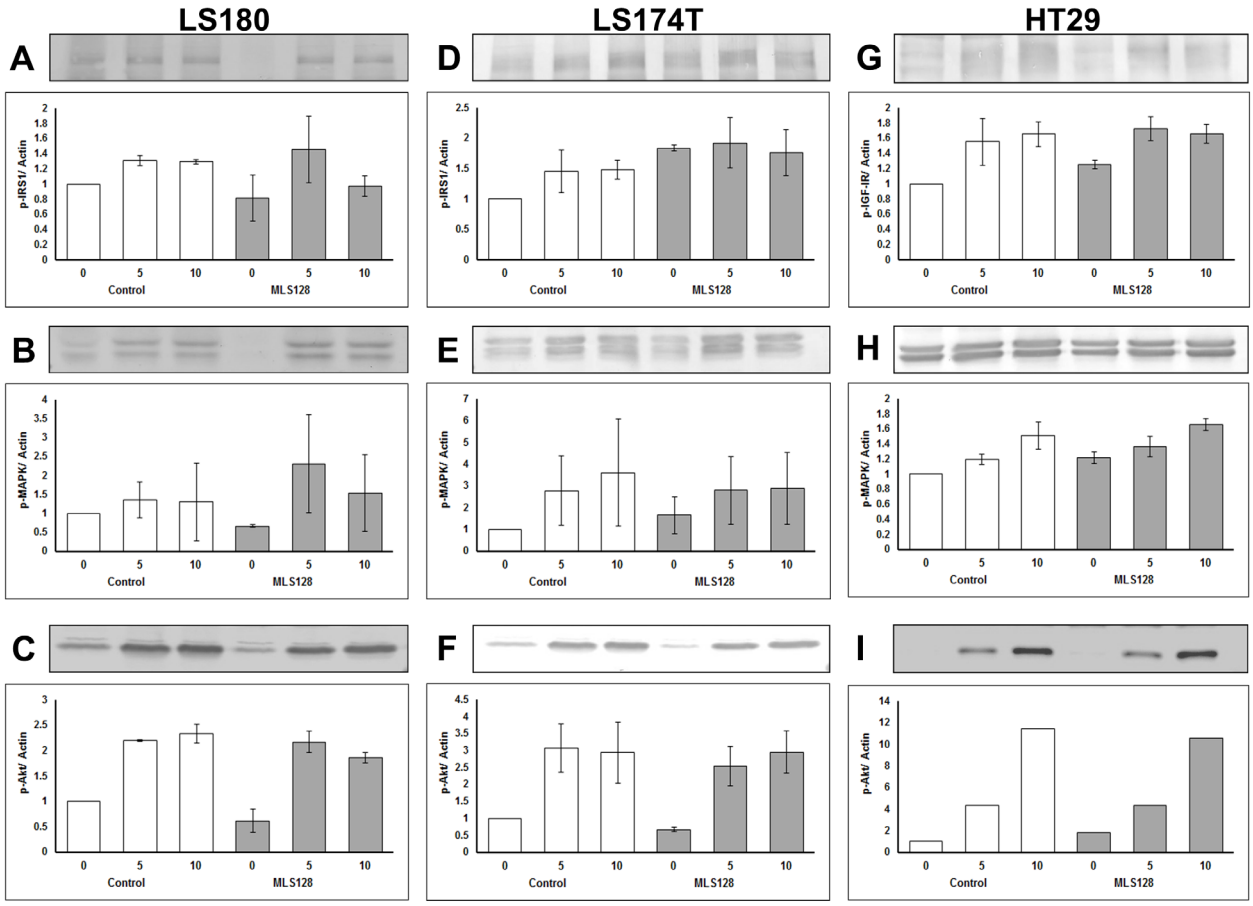

Figure 3. Effects of MLS128 on IGF-IR signaling in three colon cancer cell lines. Effects of MLS128 on IGF-IR signaling in three colon cancer cell lines were examined as described in the Methods. Briefly, 50 ng/mL of IGF-I was added to cells cultured in SFM containing 0.1% BSA (white bars) or 25 μg/mL of MLS128 (grey bars). After incubation for 5 or 10 min, cells were subjected to solubilization. The solubilized proteins (5 μg per lane) were separated by SDS-PAGE and subjected to Western blotting using primary antibodies against p-Tyr (**A**, **D**, and **G**), p-MAPK (**B**, **E**, and **H**), and p-Akt (**C**, **F**, and **I**) for detection of IRS-1, MAPK, and Akt phosphorylation, respectively. Typical immunoblots (upper panel) and average ± S.E. (n = 2-4) (lower panel) of LS180 (**A**-**C**), LS174T (**D**-**F**), and HT29 (**G**-**I**) cells are shown.

1H7 for 1, 2, and 3 days, cells were solubilized. Cell lysates were immunoblotted with anti-IGF-IR β subunit antibody to ascertain whether or not down-regulation of the IGF-IR molecules occurred in the three colon cancer cell lines after each mAb treatment.

Down-regulation of IGF-IR by 1H7 was clearly evident in all three colon cancer cell lines (Figure 4A, C, and E in the left panel). Among the three cell lines, the most significant IGF-IR down-regulation by 1H7 was noted in HT29 cells (Figure 4E, black bars). These results are consistent with the hypothesis that 1H7-induced IGF-IR down-regulation plays a role - at least in part - in inhibiting the growth of colon cancer cells.

As shown in Figure 4A, C, and E, MLS128 treatment of three colon cancer cell lines resulted in only slight down-regulation of IGF-IR in HT29 cells on day 2 and 3 (Figure 4E, grey bars), but the extent of this down-regulation paled incomparison to that induced by 1H7 (Figure 4, left panel). MLS128 treatment of LS180 cells did not cause a reduction in IGF-IR as was previously reported (*5*). Thus, the results of the current study clashed with those of a previous study indicating that MLS128 down-regulated IGF-IR in 180 cells (*5*).

3.5. *Effects of MLS128 treatment on 110 kDa GP levels in colon cancer cell lines*

The right panel in Figure 4 shows the MLS128 IB of cell lysates prepared from LS180, LS174T, and HT29 cells after treatment with MLS128 or 1H7 for 1, 2, and 3 days. Although disappearance of the 110 kDa GP band was not as apparent as was seen in IGF-IR IB, partial disappearance of the 110 kDa GP was noted in all three colon cancer cell lines when they were treated for 3 days with MLS128. Of the three colon cancer cell lines examined, however, HT29 cells treated with MLS128 had the most significant reduction in 110 kDa GP, suggesting that MLS128 binding to 110 kDa GP is possibly involved in inhibiting HT29 cell growth. In contrast, the 110 kDa GP in cells treated with 1H7 remained at the same levels as non-treated control cells (Figure 4, right panel), suggesting that 1H7-treatment had no effect on 110 kDa GP levels.

3.6. *Various levels of growth-related molecules are expressed in colon cancer cell lines*

Since previous studies by the current authors and others suggested that the growth of colon cancer cells may be mediated by IGF-IR, EGFR, and c-Met (*5,19-21*), expression of those proteins was measured in three colon cancer cell lines and control MCF-7 breast cancer cells. Western blotting analyses were carried out using two concentrations of colon or breast cancer cell lysates. Typical results of Western blots with antibodies against six molecules are shown in Figure 5.

Three colon cancer cell lines expressed the IGF-IR protein. LS174T and HT29 cells expressed the protein at a level similar to that found in MCF-7 breast cancer cells but the level of expression in LS180 cells appeared to be lower than that in other cell lines (Figure 5A). When blotted with MLS128, all three colon cancer cell lines had similar levels of 110 kDa GP but 110 kDa GP was not detected in MCF-7 cells, which instead expressed MLS128-detectable bands with higher molecular masses (Figure 5B). The MLS128 IB of cell lysates in this study confirmed previous results for MLS128 IB when cell membranes were prepared from LS180, HT29, and MCF-7 cells (*5*). All three colon cancer cell lines contained similar levels of EGFR and c-Met but MCF-7 breast cancer cells almost no expression of EGFR and c-Met (Figure 5C and D). These results suggest that different signaling pathways are used to promote growth in colon and breast cancer cells.

In addition, IB experiments were carried out using anti-E-cadherin and CD44 antibodies since E-cadherin and CD44 were reported to have a size close to that of 110 kDa GP, which MLS128 binds to (*22,23*). Interestingly, LS180 cells did not express E-cadherin whereas two other colon cancer and MCF-7 breast cancer cell lines expressed similar levels of E-cadherin (Figure 4E). Although the three colon cancer cell lines had comparable patterns of anti-CD44 reactive bands corresponding to splice variants of > 80 kDa, LS180 cells appear to express higher levels of those molecules than LS174T and HT29 cells (Figure 4F). In contrast, MCF-7 breast cancer cells expressed almost no anti-CD44 reactive bands. The results of the IB experiments are summarized in Table 1, which clearly shows that (*i*) colon and breast cancer cells express vastly different sets of growth-related molecules, and (*ii*) the three colon cancer cell lines have somewhat similar but nonetheless distinctive patterns of these molecules.

4. Discussion

This study used three established colon cancer cell lines in an effort to reveal the potential roles of IGF-IR signaling in the growth of colon cancer cells and its possible interaction with MLS128-induced inhibition of growth. The three colon cancer cell lines expressed IGF-IR. Treatment of cells with 1H7 caused inhibition of their growth and down-regulation of IGF-IR. 1H7 inhibited cell growth by 30-40%, indicating that 60-70% of cell growth must be mediated by signaling pathways other than IGF-IR signaling. MLS128 did not affect the phosphorylation of downstream signaling molecules stimulated by IGF-I such as IRS-1, MAPK, and Akt. These results suggest that MLS128-induced inhibition of the growth of colon cancer cells is independent of IGF-IR signaling. Colorectal cancer is the world's third most common cancer, and further studies are needed

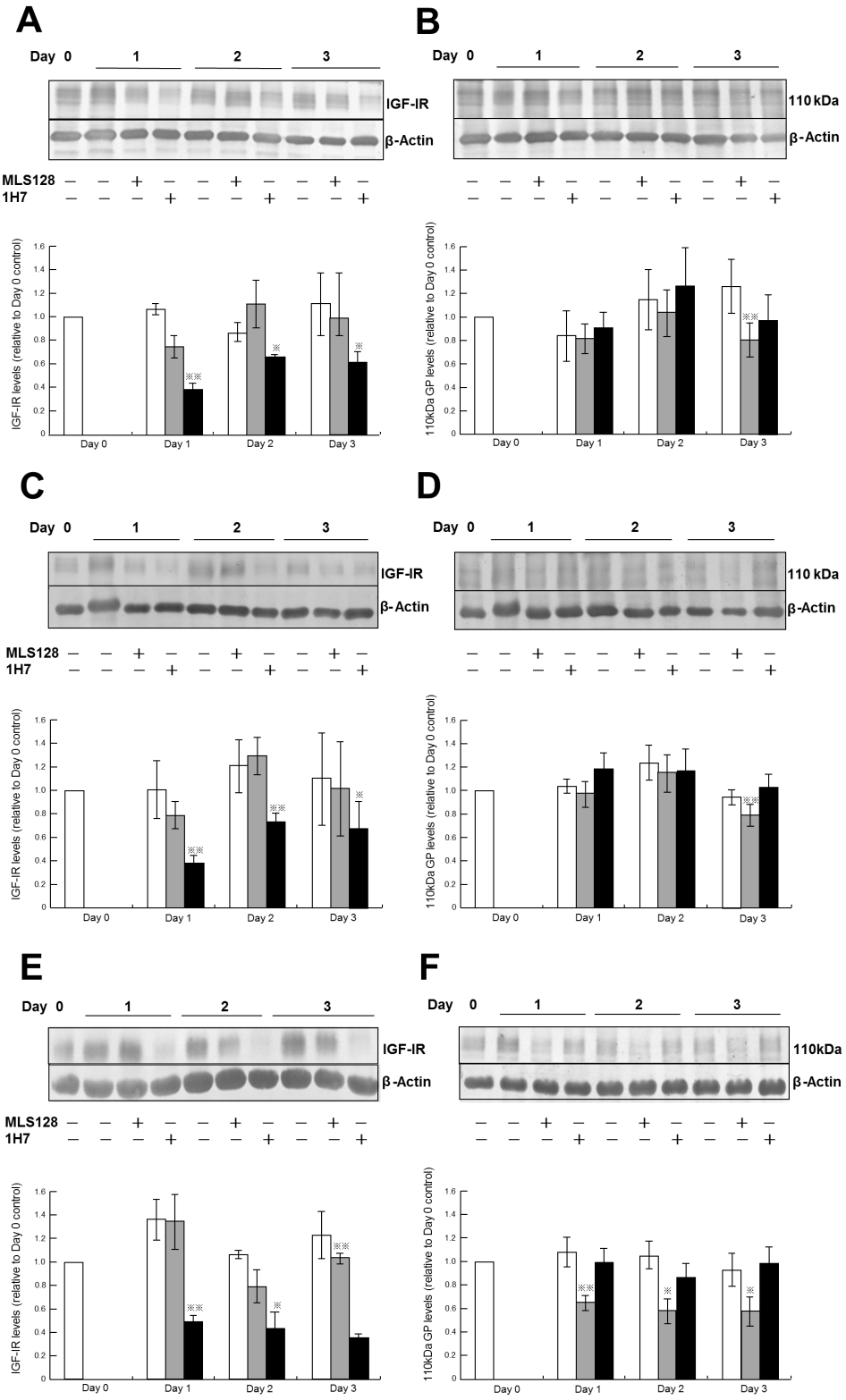

Figure 4. Effects of 1H7 or MLS128 treatment on cellular levels of IGF-IR (left panel) and 110 kDa GP (right panel). Experiments were carried out as described in the Methods, Briefly, LS180, LS174T, and HT29 colon cancer cells were cultured in respective media containing 1% FBS in the presence or absence of MLS128 (25 μg/mL) or 1H7 (0.36 μg/mL) for 24 (Day 1), 48 (Day 2) and 72 h (Day 3). Cell lysates (2 μg protein per lane) were subjected to Western blotting using primary antibodies, anti-IGF-IR β subunit, MLS128, and anti-β-actin and then subjected to color development using biotin-labeled secondary antibodies and the alkaline phosphatase kit. Shown are six panels (**A**~**F**), each of which consists of a typical immunoblot from 3-4 experiments (upper panel) and average values of IGF-IR or 110 kDa GP levels of 3 or 4 immunoblots (lower panel). The IGF-IR or 110 kDa levels were normalized to β-actin leveles. White bars represent controls, which are IGF-IR or 110 kDa GP levels from cells untreated with mAb. The grey and black bars represent IGF-IR levels (left panel) and 110 kDa GP levels (right panel) in cells treated with MLS128 and 1H7, respectively. Average values of these protein levels on Days 1, 2, and 3 were calculated with respect to Day 0. Average ± S.D. (n = 3-4) of IGF-IR (left panel) and 110 kDa GP (right panel) are shown for LS180 (**A** and **B**, respectively), LS174T (**C** and **D**, respectively), and HT29 (**E** and **F**, respectively). * $p < 0.05$; ** $p < 0.01$.

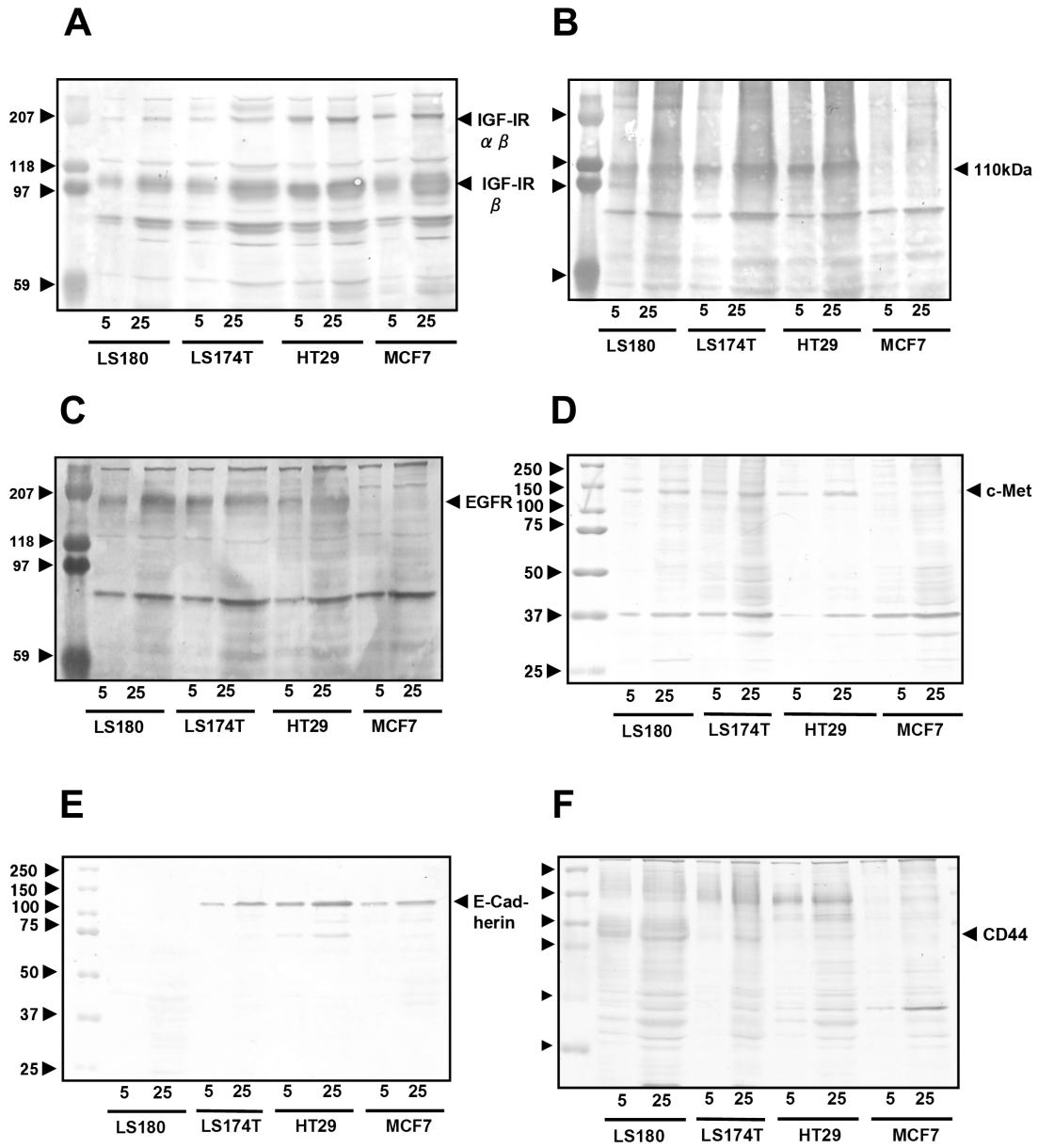

Figure 5. Different sets of growth-related molecules are expressed in colon and breast cancer cells. Expression of IGF-IR, MLS128 binding protein (110 kDa GP), and four other proteins was compared using Western blotting with 5 and 25 μg protein each of LS180, LS174T, HT29, and MCF7 cell lysates as described in the Methods. Primary antibodies used are anti-IGF-IR (**A**), MLS128 (**B**), anti-EGFR (**C**), anti-c-Met (**D**), anti-E-cadherin (**E**), and anti-CD44 (**F**). Bound primary antibodies were detected using biotin-labeled secondary antibodies for **A**, **B**, and **C**. Alternatively, bound primary antibodies were detected with HRP-conjugated secondary antibodies and color development using Ez West blue (**D**, **E**, and **F**). In contrast to relatively abundant proteins shown in A~C, proteins shown in **D**~**F** were not readily detected with biotin-labeled secondary antibodies due to the existence of non-specific 125, 75, and 73 kDa proteins functioning as endogenous biotin-containing enzymes in cells. Thus, an alternative method was used to detect sparse proteins (**D**, **E**, and **F**).

Table 1. Expression of growth-related cell surface molecules in LS180, LS174T, and HT29 colon cancer cells in comparison to MCF-7 breast cancer cells

Items	LS180	LS174T	HT29	MCF-7
IGF-IR	+	++	++	++
110 kDa GP	+	+	+	±
EGFR	+	+	+	±
c-Met	+	++	+	±
E-Cadherin	-	+	++	+
CD44	++	+	+	±

Levels of each protein are summarized from the results shown in Figure 5.

to determine the mechanisms by which its growth is inhibited. This study did not extensively examine levels of expression of growth-related molecules in colon cancers, but Figure 5 provides insight into growth signaling to each colon cancer cell line. Transcriptomes and/or proteomics could be used to examine other molecules and develop a profile of each colon cancer cell line. Such work could eventually provide a model for development of cancer therapeutics. As far as *in vivo* studies are concerned, a very recent genome-scale analysis of 276 human colon and rectal cancer samples

discovered amplification of IGF-II in addition to the expected mutations (*24*). This new finding is consistent with the results of the present study, which found that growth of colon cancer cells is IGF-dependent but only to a degree.

Western blotting analyses were carried out in an effort to narrow down other the growth signaling pathways that MLS128 may interact with (Figure 5). EGFR was expressed in all colon cancer cell lines, indicating that their growth is likely to be mediated by this receptor as well. LS180 cell growth was found to be EGF-dependent (Zamri *et al*. unpublished findings). c-Met signaling is reportedly involved in the growth of HT29 cells (*21,22*), c-Met expression in colon and breast cancer cells was determined using Western blotting. Three colon cancer cell lines expressed c-Met. These results suggest that growth of colon cancer cell lines is likely to be mediated by IGF-I, EGF, and HGF, the ligand for c-Met. Unlike the colon cancer cells, MCF-7 breast cancer cells did not express either EGFR or c-Met. The results described here are thus significant in that two colon cancer cell lines may use growth signaling similar to that in HT29 cells, which most signaling studies have thus far reported. However, their role in the growth of each cell line has to be carefully investigated since only three cell lines were compared but subtle differences were clearly noted. In fact, preliminary data indicate that the growth of LS180 cells was not stimulated by HGF (Oura *et al*. unpublished findings). More studies with established cell lines would shed light on the mechanisms of the growth of colon cancer cells and could eventually lead to the design and development of cancer therapeutics.

This study revealed one significant aspect of the nature of colon cancer cells. Namely, MLS128 binding to the 110 kD GP on colon cancer cell lines apparently resulted in inhibition of growth whereas MCF-7 cells expressed GPs with higher molecular masses as MLS128 binding proteins. In a previous study, those differences were revealed by Western blotting using membrane fractions (*5*). The current study used cell lysates for Western blotting and noted the same differences, thus suggesting that quantitative analyses of receptors and other signal molecules can be easily carried out using cell lysates instead of cell membranes. Given this, two sets of experiments were conducted as shown in Figures 4 and 5. Figure 4 shows that anti-IGF-IR antibody caused down-regulation, or more accurately speaking, degradation of the receptors in three colon cancer cell lines. In contrast, MLS128 did not down-regulate IGF-IR in colon cancer cell lines. This result rules out the original hypothesis that the down-regulation of IGF-IR is one possible mechanism by which MLS128 inhibits LS180 cell growth (*5*).

Treating three colon cancer cell lines with MLS128 for 3 days resulted in inhibition of their growth and obvious disappearance of the 110 kDa band as indicated by immunoblotting with MLS128. The disappearance of the110 kDa band was clearly evident in HT29 cells on Day 1, 2, and 3 after the mAb treatment but the disappearance of the 110 kDa band was seen only on Day 3 after the treatment of LS180 and 174T cells. The disappearance of the 110 kDa band could be explained by either the loss of the Tn-antigen epitopes on the protein backbone or degradation of the protein backbone by itself. Antibodies recognizing the protein backbone of 110 kDa GP are needed to identify whether protein degradation has occurred or Tn-antigen epitopes have been lost once colon cancer cells have been treated with MLS128. Identification of the 110 kDa GP is the next important step in producing antibodies against the 110 kDa GP and understanding MLS128's inhibition of cell growth.

In summary, growth of colon cancer cells depends in part on IGF-IR signaling as indicated by anti-IGF-IR mAb treatment inhibiting cell growth *via* IGF-IR down-regulation. The present study found that MLS128 bound specifically to 110 kDa GP in the three colon cancer cell lines examined and that IGF-IR signaling was not associated with MLS128's inhibition of the growth of LS180 cells. Results, however, suggested a possible link between IGF-IR- and 110 kDa GP-mediated growth signaling pathways in HT29 cells. The original working hypothesis regarding the interaction between two receptors has been tested further using HT29 colon cancer cells (manuscript in preparation).

Acknowledgements

This work was supported by grants (17570120 and 22570125) from the Japan Society for the Promotion of Science.

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(*Received November 24, 2012; Revised December 21, 2012; Accepted December 24, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.6.313

Effect of mild hypothermia on breast cancer cells adhesion and migration

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Summary **To explore the effect of mild hypothermia (35ºC) on breast cancer cells adhesion to vascular endothelial cells, a parallel plant flow chamber was used to observe the adhesion of human breast cancer cells MDA-MB-231 to endothelial cells Eahy926 under physiological flow at 35ºC and 37ºC, as well as the role of intercellular adhesion molecule 1 (ICAM-1) in this process. Further, the effect of mild hypothermia (35ºC) on migration of MDA-MB-231 was also studied. Our results show that mild hypothermia can inhibit the adhesion of tumor cells to endothelial cells and ICAM-1 plays an important role in this process. However, mild hypothermia inhibits breast cancer cell adhesion in a way independent on the change of ICAM-1 expression under our experimental conditions. Mild hypothermia can weaken the chemotaxis of breast cancer cells while it has no obvious effect on unidirectonal migration capacity. These results suggest that mild hypothermia could be used as a potentially adjunct treatment combined with surgery to decrease tumor cell adhesion and migration.**

Keywords: Mild hypothermia, breast cancer, cell adhesion, cell migration, intercellular adhesion molecule 1, flow chamber

1. Introduction

The metastasis of tumors is responsible for most cancer related deaths, which occurs through a series of complex processes, involving tumor cells detachment from the primary tumor, intravasation and survival in the circulation, and extravasation in the vasculature to invade the target tissue, followed by proliferation and angiogenesis at the metastasis focus (*1*). A potentially rate-limiting step in metastasis would be the extravasation process that involves tumor cells arrest or adhesion to endothelium ("docking"), transition to more established cell contacts and numerous focal adhesions ("locking") and transmigration through the endothelial cell monolayer and basement membranes (*2*). Various endothelial adhesion molecules (including E-selection, P-selection, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), β1 integrin, N-cadherin, *etc*.) contribute to tumor cells extravasation (*2-4*). ICAM-1 is widely expressed at a low basal level and is upregulated by inflammatory cytokines (TNF-α, IL-1, IFN-γ) in endothelial cells. In various cancers, such as non-small cell lung cancer (*5*), breast cancer (*6*), gastric cancer (*7*), colorectal cancer (*8*), and bladder cancer (*9*), ICAM-1 expression is at an elevated level and is associated with the malignant potential of cancer. Increasing evidence suggest that ICAM-1 plays an important role in the adhesion of tumor cells to endothelial cell monolayers and subsequent transendothelial migration (*10-13*). Adherence of human small-cell lung carcinoma to cultured vascular endothelial cells in stasis and flow depends on the expression of endothelial ICAM-1 (*10*). In addition, endothelial ICAM-1 supports breast cancer cells adhesion *via* the ligand mucin-1 (MUC-

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1), a transmembrane glycoprotein expressed by normal breast epithelium and virtually all breast cancers. Tumor binding to endothelial ICAM-1 *via* MUC-1 initiates a calcium signal and triggers Src family kinase-mediated cell motility, and eventually promotes transendothelial migration (*11-13*). Furthermore, leukocytes act as bridge-cells and facilitate the contact between tumor cells and the endothelium in which ICAM-1 plays an important role (*14-16*). *In vivo*, total body irradiation triggers tumor cell extravasation and lung metastasis in Balb/c mice accompanied by upregulation of both E-selectin and ICAM-1 mRNA levels and activation of nuclear factor-κB (NF-κB) in large lung blood vessels (*17*). Lovastatin impairs irradiation induced upregulation of both E-selectin and ICAM-1 and attenuates irradiation induced metastasis (*17*). All of these data imply that ICAM-1 plays a vital role in tumor adhesion to endothelial cells and extravasation.

Although many prevention methods such as chemoprevention, risk-reduction surgery, molecular genetics, epidemiology, and imaging have been used to reduce the incidence of cancer, few have paid attention to mild hypothermia (32-35ºC) as a strategy to prevent tumor cell adhesion and extravasation although mild hypothermia (32-35ºC) has been studied to improve outcome from cardiac arrest, brain trauma, stroke, hypoxic encephalopathy of neonates, spinal cord injury, open cardiac surgery, and other ischemic tissue injury for a long time (*18*). One of the important mechanisms is that mild hypothermia inhibits the inflammation response which contributes significantly to secondary injury after ischemia (*19,20*). Mild hypothermia suppresses ICAM-1 overexpression and neutrophil accumulation following acid-induced lung injury (*21*) and experimental stroke (*20,22*). Suppression of ICAM-1 induction by mild hypothermia is mediated by enhanced ERK1/2 activation and subsequent attenuation of STAT3 action (*23*). In addition, mild hypothermia can protect against TNF-α-induced endothelial barrier dysfunction and apoptosis through an MKP-1-dependent mechanism (*24*). Besides the effect on the expression of ICAM-1, mild hypothermia may also influence the binding thermodynamics of tumor cells to endothelial cells. In all cases of adhesion of cells to other cells or to extracellular matrix (ECM), noncovalent receptor-ligand interactions are a prerequisite for firm adhesion. The receptor-ligand binding mainly depends on the size, structure, formational dynamics and environmental temperature of their binding sites. In many cases, receptor-ligand binding can be treated as a reversible reaction. Both the association rate and dissociation rate are affected by temperature (*25-28*).

All these findings described above identify that mild hypothermia can be a potential method to reduce cancer adhesion and extravasation and then can prevent cancer metastases. The objective of the present study is to test the hypothesis that mild hypothermia can reduce breast tumor cells adhesion to endothelial cells and to try to investigate the potential mechanism. To reduce hypothermia-associated adverse side effects in the clinic and reduce the difficulty to achieve mild hypothermia, only 35ºC was chosen as the mild hypothermia to test this hypothesis in our study.

2. Materials and Methods

2.1. *Cell culture*

Human breast cancer cells MDA-MB-231 (from the Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming, China) were cultured in high-glucose DMEM medium (Life Technologies, Carlsbad, CA, USA) with 10% FBS (TBD, Tianjin, China), 100 UI/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 26 mM $NaHCO_3$ in a humidified 95% air/5% CO_2 environment at 37ºC. Human breast cancer cells MCF-7 (from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in low-glucose DMEM medium (Life Technologies) with 10% FBS. Endothelial cells (ECs) Eahy926 (from ATCC, USA) were grown in RPMI1640 medium (Fisher Scientific, Beijing, China) containing 10% FBS in the same conditions. At 80-90% confluence, cells were trypsinized (0.25% trypsin and 0.02% EDTA) and passaged at a 1:5 ratio.

2.2. *Preparation of parallel-plate flow chamber*

The structure and working principle of the flow chamber have been described in detail (*29*). In brief, the parallel plate flow chamber consisted of a parallel plate flow chamber, silicone tubes, a peristaltic pump, and a reservoir. The parallel plate flow chamber is composed of two silicone shims (B_1 and B_2), three plexiglass plates (C_1, C_2 and C_3), and a glass slide coated with cells, with the depth (d) of the chamber (A) being 300 μm, the width (b) 2.5 cm, and the length (l) 7.5 cm (Figure 1). Both the liquid inlet D and the outlet G were linked to the tubes (H_1 and H_2) on the first plexiglass plate C_1. The triangle narrow slit E in silicone insole B_1 can distribute the liquid uniformly and reduce pulsation. Then, the liquid goes into the chamber through rectangular slit F. The glass slide coated with the endothelial cells is placed into the chamber and perfused with medium containing tumor cells. Under fully developed laminar flow, the shear stress (τ) on the cell monolayer can be calculated by $\tau = 6\mu Q/bd^2$ dyn/cm^2, where Q is the volumetric flow rate (cm^3/sec), μ is the viscosity of the circulating medium (dyn•sec/cm^2), b is the width of chamber and d is the depth of the chamber. In our experiments, the viscosity of the medium μ was chosen as 0.012 dyn•sec/cm^2 (*30*) and the shear stress τ was set at 1.5 dyn/cm^2 by changing the volumetric flow rate Q (*31*).

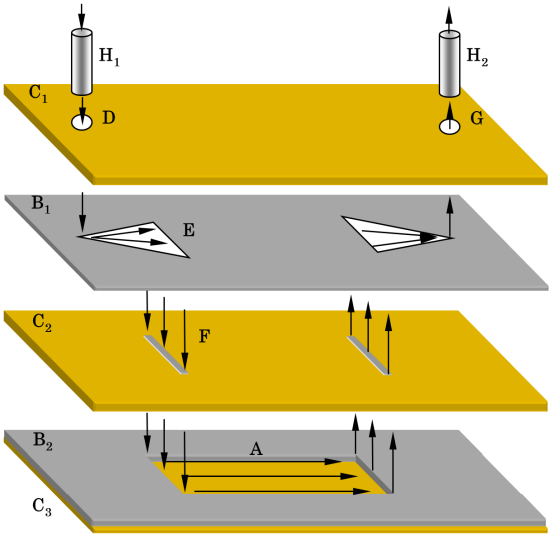

Figure 1. Sketch of parallel-plate flow chamber. A is the flow chamber (l = 7.5 cm, b = 2.5 cm, d = 0.3 mm), B_1 and B_2 are the two silicone shims with 0.5 mm and 1.5 mm depths, respectively. C_1, C_2 and C_3 are the plexiglass plates with 4 mm, 4 mm and 2 mm depths. D is the liquid inlet and G is the liquid outlet. E is the triangle narrow slit, F is the rectangular slit. H_1 and H_2 are the tubes made from polytetrafluoroethylene.

2.3. *Flow chamber adhesion assays*

At 80% confluence, the ECs were trypsinized (0.25% trypsin and 0.02% EDTA), and seeded on a glass microscope slide (75 mm × 25 mm) at a cell concentration of 10^5 cells/mL. The ECs could be confluent in two days after seeding. The slide was attached to the bottom of the flow chamber before the flow experiment. The MDA-MB-231 cells were trypsinized and stained with 2 μM Calcein-AM (C3099, Molecular Probes, Life Technologies) for 15 min at 37ºC. After washing twice with PBS, the MDA-MB-231 cells were resuspended at 5×10^5 cells/mL in high-glucose DMEM medium with 1% FBS, and incubated at 37ºC for 1 h before perfusion. First, the incubator was adjusted to 35ºC or 37ºC. The chamber was circulated with cell-free DMEM medium at 1.5 dyn/cm^2 for 20 min to balance the ECs and sweep the loose ECs away and to keep the parallel-plate flow chamber at 35ºC or 37ºC. Then medium with 5×10^5 cells/mL MDA-MB-231 cells was circulated at 1.5 dyn/cm^2 for 20 min. Finally, the chamber was perfused at 1.5 dyn/cm^2 for 5 min with cell-free medium to remove nonadherent cancer cells. In these assays, perfusions were done both at 37ºC and 35ºC and repeated three times at each temperature. Adherent MDA-MB-231 cells were counted with a fluorescence microscope (IX71, Olympus, Tokyo, Japan) (more than 10 fields for each slide at ×40).

Cell death rate of MDA-MB-231 cells was verified using trypan blue (Sigma-Aldrich, St. Louis, MO, USA) before and after the experiment. The live cell rate was about 97% prior to the experiment while it was about 96% after 20 min perfusion. This implies that the MDA-MB-231 cells can maintain a high survival rate in the parallel-plate flow chamber under flow condition for 20 min.

For antibody blocking assays, an endothelial monolayer on a glass slide was incubated at 37ºC for 30 min with an anti-ICAM-1 antibody (bs-0608R, Bioss, Beijing, China) (final concentration: 10 μg/mL) before perfusion at 37ºC.

Our experimental results showed that MCF-7 hardly adhered to the Eahy926 monolayer under the same conditions used for MDA-MB-231 cells flow chamber adhesion assays described above (data not shown). Thus, a detachment assay (*32*), which was a little different from the MDA-MB-231 cells adhesion assay, was carried out inside the parallel plate flow chamber to investigate the effect of mild hypothermia on adhesion of MCF-7 cells to ECs. In brief, the chamber was circulated with cell-free DMEM medium at 1.5 dyn/cm^2 for 20 min to balance the ECs, to sweep the loose ECs away, and to keep the parallel-plate flow chamber at 35ºC or 37ºC. Medium with 5×10^5 cells/mL MCF-7 cells stained by Calcein-AM was introduced and circulated at 1.5 dyn/cm^2 for 10 min. Then, the flow was stopped and MCF-7 cells were allowed to settle and adhere to the endothelial monolayer for a period of 10 min. Finally, the chamber was perfused with cell-free medium at 1.5 dyn/cm^2 for 5 min to remove nonadherent cancer cells. The adherent MCF-7 cells were also counted under a fluorescence microscope.

2.4. *Western blotting for ICAM-1*

ECs plated on a glass slip were perfused for 45 min in the flow chamber with cancer cells-free medium at 37ºC and 35ºC at 1.5 dyn/cm^2. Then, treated cells were lysed with RIPA buffer containing a protease inhibitor cocktail. Equal amounts of cell lysates were separated using 5-10% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The transferred membrane was blocked with blocking buffer at room temperature (RT) for 1 h followed by being immunoblotted with anti-human ICAM-1 antibody (dilution 1:250, sc-8439, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4ºC overnight, washed in TBST for 5 min each time and repeated twice. The same membrane was reprobed with monoclonal anti-β-actin antibody (dilution 1:500, sc-47778, Santa Cruz Biotechnology). The membrane was immunoblotted with appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at RT. The membrane was subsequently washed in washing buffer three times, 5 min for each time, and then visualized with a DAB kit (ST033, Beyotime Biotech, Haimen, China). A densitometric measurement was made from the film using a GS-800

imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA). To quantify the relative protein expression, the optical density of the protein band of ICAM-1 was quantified by Quantity One software (Bio-Rad Laboratories) and normalized to the optical density of β-actin on the same membrane.

2.5. *Cancer cells adhesion to ICAM-1-coated glass slide*

To improve protein adsorption, glass slips for ICAM-1 mobilization were silanized with 3-aminopropyltrimethoxysilane (Sigma-Aldrich) as described (*33*). Briefly, glass slips were cleaned by overnight immersion in 20% sulfuric acid, washed with copious amounts of running tap water for 20 min and with distilled water three times, then rinsed with 95% ethanol and dried overnight. The cleaned slips were treated with 0.1 M sodium hydroxide and were blotted dry. 3-Aminopropyltrimethoxysilane (4% v/v in anhydrous acetone) was layered onto one side of each slip ensuring complete coverage. After air drying, the silane-treated slips were washed three times with PBS. Recombinant human ICAM-1 (Cat#150-05, PeproTech, Rocky Hill, NJ, USA) in PBS (5 μg/mL, 0.5 mL) was pipetted onto the treated surfaces of the slips which were allowed to incubate overnight at 4ºC. The slips were washed using PBS and further incubated with 1% BSA for 1 h at RT, then stored at 4ºC under PBS until flow chamber assays. An ICAM-1-coated slip was placed into the flow chamber and perfused with medium containing with 5×10^5 cells/mL MDA-MB-231 cells for 20 min at 37ºC and 35ºC. The following steps were the same as the assay of MDA-MB-231 cells adhesion to endothelial cells described in Section 2.3.

2.6. *Cell migration*

Wound healing assays (non-oriented migration): cancer cell monolayers in 6 wells were disrupted to generate a cross wound with a pipette tip (1,000 μL). The cultures were washed three times with PBS to remove floating cells and then incubated at 37ºC or 35ºC in DMEM medium with 10% FBS and mitomycin C (1 μg/mL) (Sigma-Aldrich). To evaluate 'wound closure' under different temperature conditions, four selected areas close to each cross wound were first photographed under phase contrast microscopy at 0 h. Cells were stained with DAPI after 36 h and followed by photography under the fluorescence microscope to count cells invading the artificial wound.

Transwell migration assays (oriented migration): 1.5 mL DMEM medium within 3×10^5 MDA-MB-231 cells stained with Calcein-AM (Life Technologies) were plated in the top chamber (6-well insert; pore size, 8 μm; Millipore, Billerica, MA, USA). Medium supplemented with 10% FBS was used as a chemoattractant in the lower chamber. The cells were incubated for 6 h at 37ºC or 35ºC and cells that did not migrate through the pores were removed using a cotton swab. Cells on the lower surface of the membrane were counted in 5 fields (×40) per well under a fluorescence microscope. Stromal cell-derived factor-1 (SDF-1) plays an important role in chemotaxis of cancer cells and in tumor metastasis (*34*). To investigate the effect of mild hypothermia on MDA-MB-231 cells migrating to a specific chemoattractant, 40 ng/mL SDF-1 in DMEM medium with 1% FBS was also added in the lower chamber and cancer cells in DMEM medium with 1% FBS were plated in the top chamber, then incubated for 6 h at 37ºC or 35ºC.

2.7. *Statistical analysis*

In our experiments, adherent cell numbers were measured at 20 min and expressed as cellnumber per cm^2 or mm^2 plane area of a calculated segment. Each experiment was performed at least three times. All values in the text are means ± S.E. (standard error). Comparisons between groups were made by one-way ANOVA. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. *Effect of mild hypothermia on tumor cell adhesion to ECs*

Figure 2 illustrates the adhesion of MDA-MB-231 tumor cells to ECs monolayer under flow condition (1.5 dyn/cm^2) at normothermia (37ºC), mild hypothermia (35ºC) and pretreated ECs with anti-ICAM-1 antibody at 37ºC. Figures 2A-C show the photomicrographs of adhesion of MDA-MB-231 tumor cells to ECs at 20 min in the flow chamber at normothermia (37ºC), at normothermia (37ºC) after pretreated ECs with anti-ICAM-1 antibody and at mild hypothermia (35ºC). The adherent MDA-MB-231 cells were the white points under the fluorescence microscope after perfusion with cell-free medium for 5 min. Under normothermia (37ºC), 493.92 (± 72.70 S.E., $n = 3$) cells/cm^2 adhered firmly to the ECs under 1.5 dyn/cm^2 shear stress. After 20 min, mild hypothermia (35ºC) significantly decreased the adherent MDA-MB-231 cells from 493.92 (± 72.70 S.E., $n = 3$) to 50.22 (± 4.01 S.E., $n = 3$) cells/cm^2 ($p < 0.01$) (Figure 2D). The results of cell adhesion suggested that mild hypothermia (35ºC) can attenuate MDA-MB-231 cell adhesion to ECs. To study whether the adhesion of MDA-MB-231 tumor cells to ECs was mediated by ICAM-1, adhesion experiments were further performed after incubating ECs with mAb against ICAM-1. Pretreatment of the ECs with anti-ICAM-1 antibody significantly prevented the adhesion of tumor cells under experimental shear stress

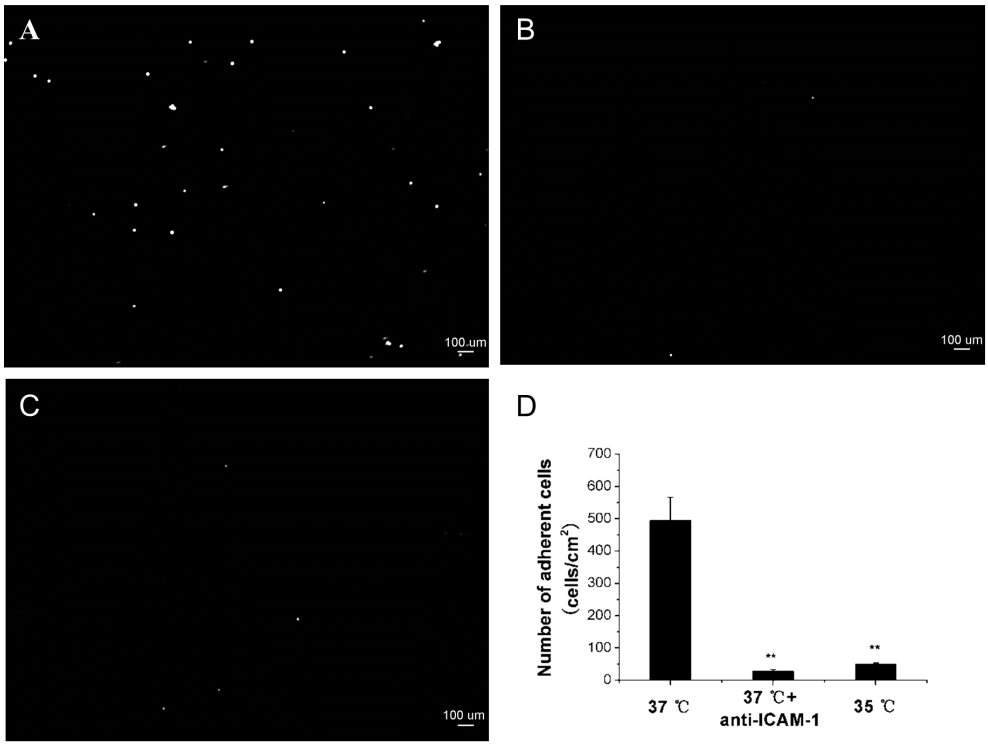

Figure 2. Effect of the mild hypothermia (35ºC) and anti-ICAM-1 on the adhesion of MDA-MB-231 tumor cells to Eahy926 endothelial cells under flow (1.5 dyn/cm^2). (A-C), Photomicrographs of adhesion of MDA-MB-231 tumor cells to Eahy926 endothelial cells under flow (1.5 dyn/cm^2) at normothermia (37ºC) **(A)**, pretreatment of the endothelium with anti-ICAM-1 antibody at 37ºC **(B),** and at mild hypothermia (35ºC) **(C)**. The white points were adherent tumor cells. The images were taken by with a fluorescence microscope (×40). **(D)** Adherent MDA-MB-231 cells were counted with a fluorescence microscope (more than 10 fields for each slide at ×40). Values are the mean of three experiments (n = 3) and are means ± S.E. ** $p < 0.01$ Comparison of adhesion at mild hypothermia (35ºC) or anti-ICAM-1treatments with the control treatment (37ºC).

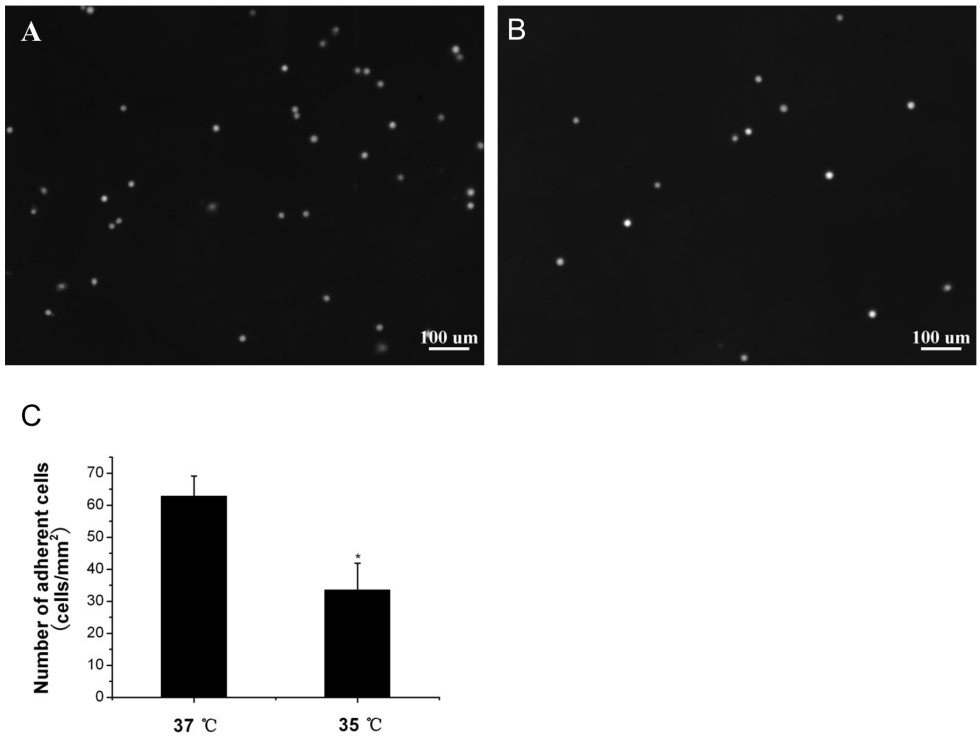

Figure 3. Effect of the mild hypothermia (35ºC) on the adhesion of MCF-7 tumor cells to Eahy926 endothelial cells under flow (1,5 dyn/cm^2). (A, B) Photomicrographs of adhesion of MCF-7 tumor cells to Eahy926 endothelial cells under flow (1.5 dyn/cm^2) at normothermia (37ºC) **(A)** and mild hypothermia (35ºC) **(B)**. The white points were adherent tumor cells. The images were taken with a fluorescence microscope (×100). **(C)** Adherent MCF-7 cells were counted with a fluorescence microscope (more than 10 fields for each slide at ×100). Values are the mean of three experiments and are means ± S.E. * $p < 0.05$ Comparison of adhesion at mild hypothermia (35ºC) with the control treatment (37ºC).

levels and only 28.35 (± 4.03 S.E., n = 3) cells/cm^2 firmly adhered to the endothelium at 37ºC ($p < 0.01$) (Figure 2D). This indicated that ICAM-1 might play an important role in the adhesion of MDA-MB-231 tumor cells to ECs. Because the number of adherent MDA-MB-231 cells in the field under the fluorescence microscope at ×40 was very few after pretreatment with mild hypothermia (35ºC), the effect of anti-ICAM-1 antibody on tumor cell adhesion under mild hypothermia (35ºC) was not further observed. Our results indicated that both mild hypothermia and anti-ICAM-1 antibody treatments significantly decreased adhesion of MDA-MB-231 tumor cells to ECs under flow conditions (1.5 dyn/cm^2).

To verify whether the mild hypothermia function is a general phenomenon in breast cancer cell lines, a detachment assay was used to investigate the effect of mild hypothermia on MCF-7 cells adhesion to ECs. The number of adhesion MCF-7 cells was decreased from 62.88 (± 6.23 S.E., n = 3) to 33.53 (± 8.32 S.E., n = 3) cells/mm^2 ($p < 0.05$) (Figure 3), which implied that mild hypothermia also has a similar effect on MCF-7 cells adhesion to ECs.

3.2. *Effect of mild hypothermia on the expression of ICAM-1 on Eahy926 endothelial cells*

As shown in Figures 2 and 3, mild hypothermia (35ºC) could reduce the MDA-MB-231 and MCF-7 tumor cells adhesion to ECs and ICAM-1 might play an important role in this process. Quantitative measurement of ICAM-1 expression on ECs was performed using Western blot analysis to examine the effect of the mild hypothermia on the expression of ICAM-1. Pretreatment of the ECs with mild hypothermia (35ºC) for 45 min in the flow chamber did not change the expression of ICAM-1 significantly (p = 0.325) (Figure 4). These results imply that mild hypothermia may decrease tumor cell adhesion by other mechanisms but not by decreasing the expression of ICAM-1 on ECs.

3.3. *Effect of mild hypothermia on tumor cells migration*

To determine whether mild hypothermia can affect the migration ability of tumor cells *in vitro*, wound healing assay (non-oriented migration) and transwell migration assays (oriented migration) were performed. In standard wound healing assays, the numbers of migrating MDA-MB-231 cells in a 100 μm long wound line were 25.57 (± 3.98 S.E., n = 3) and 23.62 (± 4.18 S.E., n = 3) cells under normothermia (37ºC) and mild hypothermia (35ºC), respectively (Figure 5). However, there is no obvious difference between the migrating cell numbers at normothermia (37ºC) and mild hypothermia (35ºC) ($p > 0.05$) (Figure 5B). Without mitomycin C treatment, the number of tumor cells in the 100 μm long wound

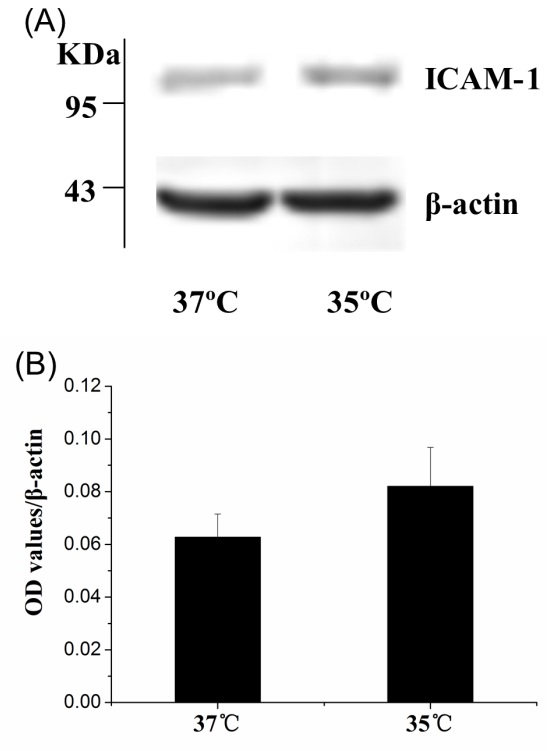

Figure 4. Effect of mild hypothermia on the expression of ICAM-1 on Eahy926 endothelial cells in the flow chamber. (A) Results of Western blotting. **(B)** Results of relative optical density values. The Eahy926 endothelial cells were perfused for 45 min in the flow chamber at 1.5 dyn/cm^2 with cancer cell-free medium at 37ºC and 35ºC. The expression of ICAM-1 showed no significant difference between 37ºC and 35ºC. Relative optical density values are the mean of three experiments (n = 3) and are means ± S.E.

line at normothermia (37ºC) were more than that at mild hypothermia (35ºC) for 36 h (data not shown). Temperature affects the growth of tumor cells without mitomycin C treatment.

In transwell migration assays, 6 h mild hypothermia treatment decreased the MDA-MB-231 cells oriented migration from 37.26 (± 2.85 S.E., n = 3) to 18.67 (± 3.37 S.E., n = 3) cells/visual field significantly ($p < 0.05$) and from 122.17 (± 11.65 S.E., n = 3) to 64.08 (± 11.03 S.E., n = 4) cells/visual field significantly ($p < 0.05$), using 10 % FBS and 40 ng/mL SDF-1 as chemoattractant, respectively (Figure 6). These results suggested that the effects of mild hypothermia on non-oriented migration and oriented migration of MDA-MB-231 cells were different. Mild hypothermia (35ºC) was able to weaken the oriented migration of MDA-MB-231 cells, but was not able to weaken the non-oriented migration.

3.4. *Effect of mild hypothermia on tumor cell adhesion to ICAM-1-coated substrate*

The above experimental results showed that mild hypothermia could reduce adhesion of tumor cells to the ECs, but could not reduce the expression of ICAM-

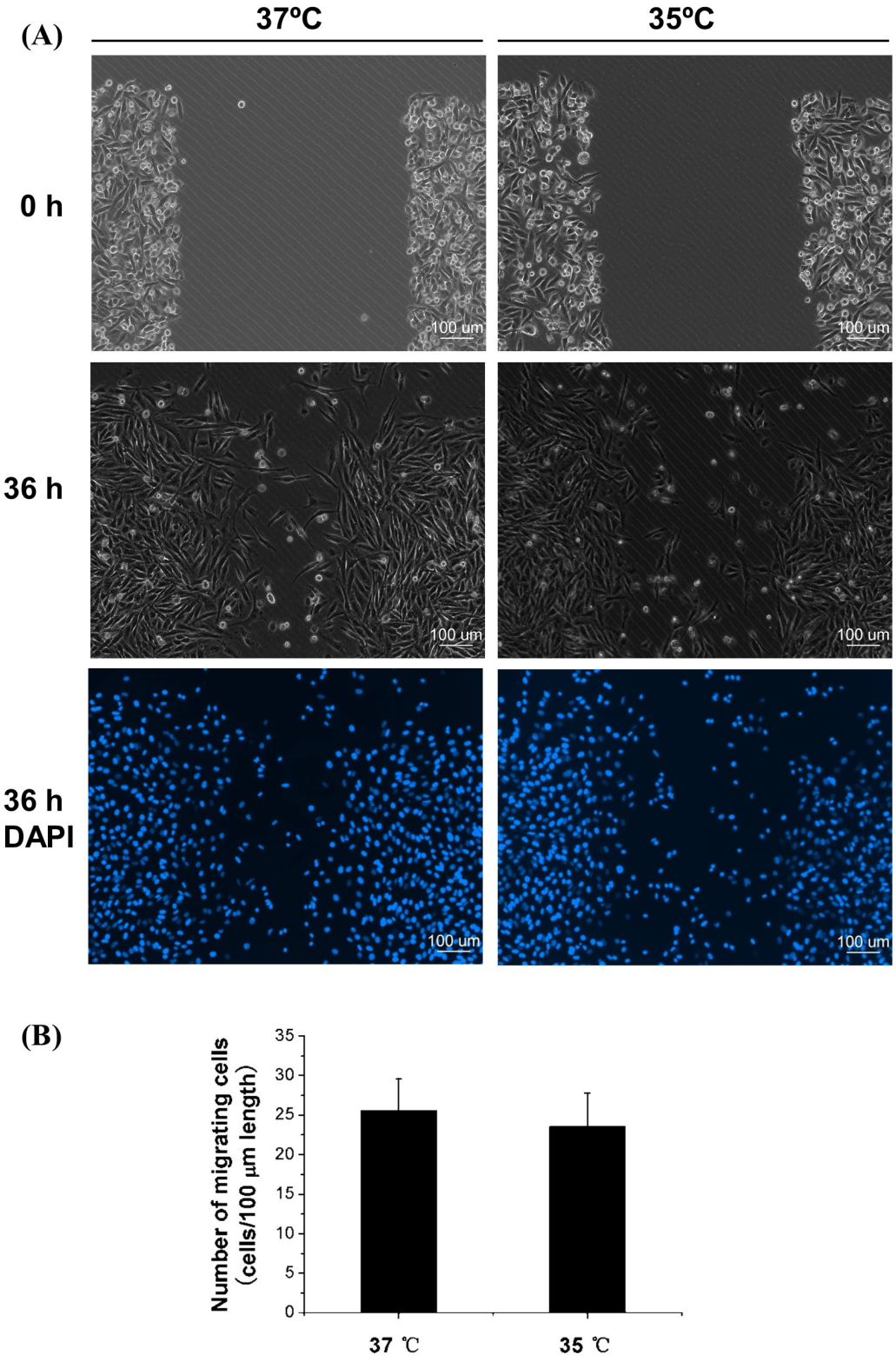

Figure 5. Effects of mild hypothermia on MDA-MB-231 tumor cells non-oriented migration in wound healing assay. (A) The images at 0 h and 36 h (the first and second rows) were taken using phase contrast microscopy (×100). The images at the bottom row were the same fields as those at 36 h and the cells were stained with DAPI, which were taken using a fluorescence microscope (×100). **(B)** Number of non-oriented migrating MDA-MB-231 tumor cells at 36 h in wound healing assay at normothermia (37ºC) and mild hypothermia (35ºC). The number of migrating cells was counted in 100 μm long wound line. Values are the mean of three experiments (n = 3) and are means ± S.E.

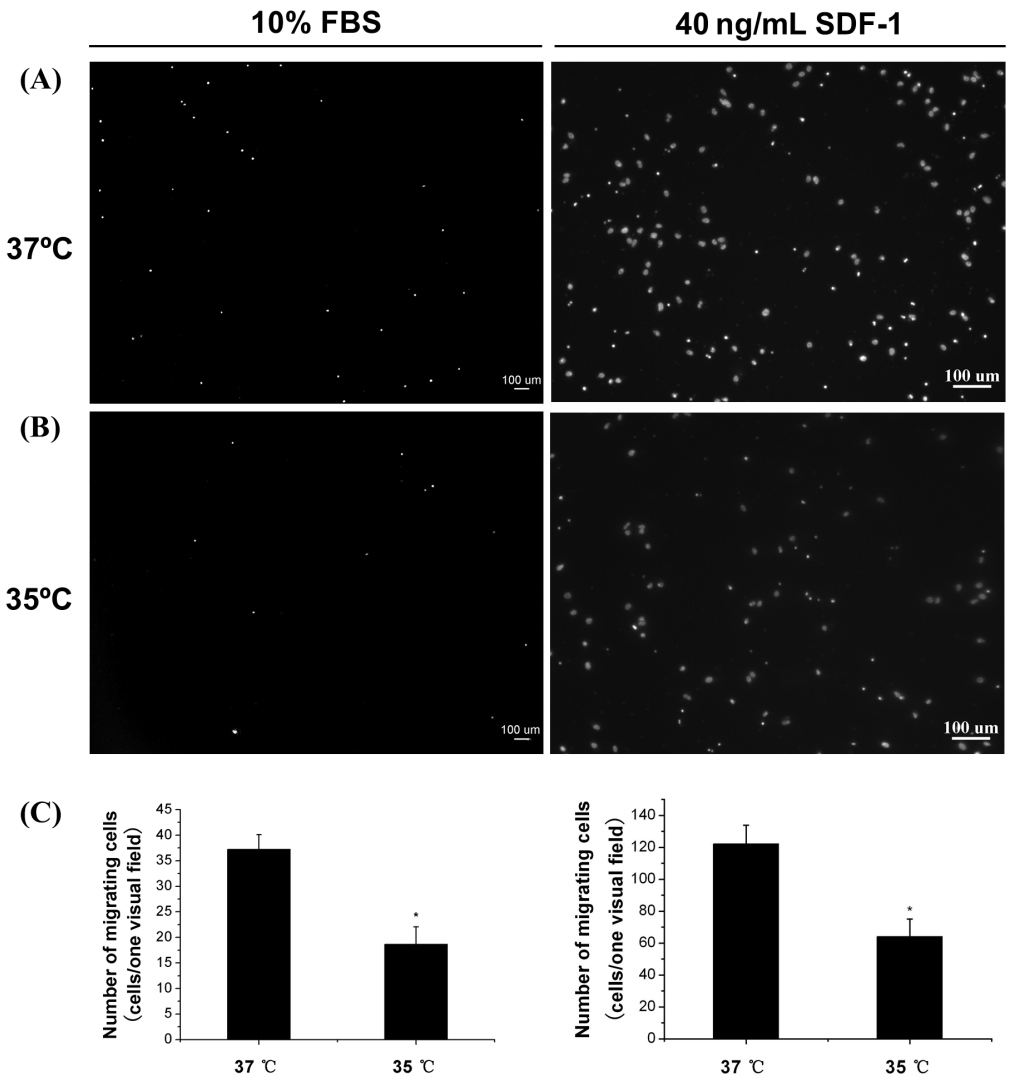

Figure 6. Effects of mild hypothermia on MDA-MB-231 tumor cells oriented migration in transwells migration using 10% FBS and 40 ng/mL SDF-1 as chemoattractant. (A, B) Photomicrographs of MDA-MB-231 tumor cells oriented migration in transwells migration assay at normothermia (37ºC) **(A)** and mild hypothermia (35ºC) **(B)**. The white points were adherent tumor cells. The images were taken using a fluorescence microscope (×40 for left and ×100 for right). **(C)** Number of oriented migrating MDA-MB-231 tumor cells in transwell migration assay at normothermia (37ºC) and mild hypothermia (35ºC). The number of migrating cells was counted in one visual field under a fluorescence microscope (×40 for FBS and ×100 for SDF-1). Values are the mean of at least three experiments and are means ± S.E. * $p < 0.05$ Comparison of migrating cells in transwells migration assays at normothermia (37ºC) and mild hypothermia (35ºC).

1 on ECs. It is indicated that the decreasing of tumor cells adhesion to ECs under mild hypothermia was not due to decreased expression of ICAM-1 on ECs, although ICAM-1 played an important role in the adhesive process. Tumor cells adhesion to the ICAM-1-coated substrate under flow conditions (1.5 dyn/cm^2) was further performed at normothermia (37ºC) and mild hypothermia (35ºC). 138.01 (± 19.18 S.E., $n = 3$) cells/mm^2 MDA-MB-231 cells adhered firmly to the ICAM-1-coated substrate under flow at 37ºC, while the adhesion was significantly reduced and only an average of 21.50 (± 8.15 S.E., $n = 3$) cells/mm^2 MDA-MB-231 cells adhered at 35ºC ($p < 0.01$) (Figure 7). These results further confirmed that mild hypothermia (35ºC) was able to reduce the adhesion of MDA-MB-231 tumor cells to ECs by a way independent of the change of ICAM-1 expression.

4. Discussion

The above results proved that minimal mild hypothermia (35ºC) was able to significantly reduce adhesion of MDA-MB-231 tumor cells to ECs Eahy926 under physiological flow *in vitro*. This finding implies that mild hypothermia can effectively reduce the adhesion of tumor cells to endothelium while the effective temperature is slightly lower than physiological temperature, 35ºC *versus* 37ºC. A deeper level of mild hypothermia such as 33ºC was widely used to study protection of mild hypothermia in ischemic tissue injury

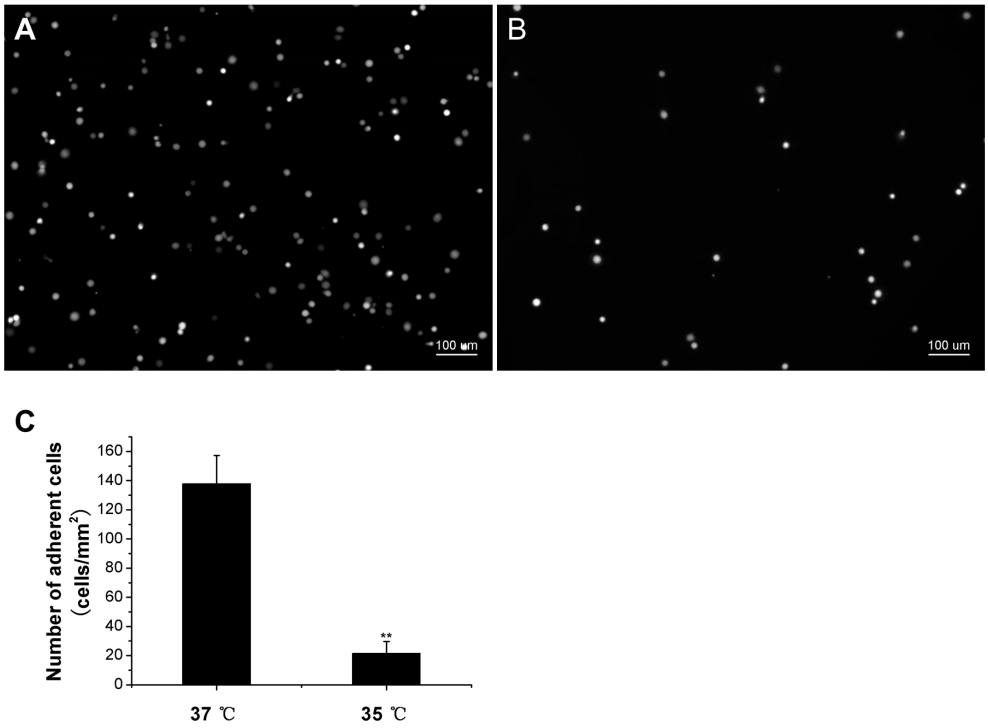

Figure 7. Effects of mild hypothermia on MDA-MB-231 tumor cells adhesion to ICAM-1-coated substrate under flow (1.5 dyn/cm^2). (A, B) Photomicrographs of MDA-MB-231 tumor cells adhesion to ICAM-1-coated substrate under flow (1.5 dyn/cm^2) at normothermia (37ºC) **(A)** and mild hypothermia (35ºC) **(B)**. The white points were adherent tumor cells. The images were taken using a fluorescence microscope (×100). **(B)** Number of adherent of MDA-MB-231 tumor cells to ICAM-1-coated substrate at 20 min at normothermia (37ºC) and mild hypothermia (35ºC). The number of migrating cells was counted in mm^2. Values are the mean of three experiments (n = 3) and are means ± S.E. ** $p < 0.01$ Comparison of adhesion at mild hypothermia (35ºC) with the control treatment (37ºC).

models and in clinical trials (*35,36)*. In comparison to 33ºC, 35ºC is easier to achieve in the clinic and better to tolerate. In addition, the hypothermia-associated adverse side effects might be less.

In blocking assays, treatment of ECs with anti-ICAM-1 antibody significantly prevented adhesion of tumor cells under experimental shear stress level. The result indicated that ICAM-1 might play an important role in the adhesion of MDA-MB-231 tumor cells to ECs Eahy926 which was consistent with previous studies (*10,37*). But Western blotting assays showed that mild hypothermia did not immediately reduce endothelial ICAM-1 expression under our experimental conditions. Thus, the reduced adhesion of MDA-MB-231 tumor cells to ECs Eahy926 under mild hypothermia (35ºC) is not due to a decrease of ICAM-1 expression by mild hypothermia (35ºC) in our study. ICAM-1 is expressed constitutively on endothelial cells and induced overexpression is caused by inflammatory cytokines (TNF-α, IL-1, IFN-γ). It is reported that mild hypothermia (33ºC) treatment inhibited the overexpression of ICAM-1 after acid-induced lung injury in rats, whereas it did not significantly affect express of ICAM-1 in normal rats (*21*). Sutcliffe and his colleagues (*38*) reported that ICAM-1 expression has no significant difference when human cerebral endothelial cells were exposed to IL-1β at 37ºC and 32ºC. However, a significant reduction in IL-8 and IL-1β mRNA expression was seen in human cerebral endothelial cells exposed to IL-1β at 32ºC. In contrast to IL-1β, TNF-α is increased after application of hypothermia (32ºC) 8 h after the insult in an endothelin-1-induced transient focal cerebral ischemia model (*19*). Therefore, it is likely that the inhibition of ICAM-1 overexpression by mild hypothermia in injury models is a cumulative outcome due to different effects of mild hypothermia on individual inflammatory factors (such as TNF-α and IL-1β), transcription factors involved in inflammation (such as NF-κB and signal transducer and activator of transcription-3 (STAT3)). A role of inflammation in tumorigenesis is now generally accepted. There are several types of inflammation associated with cancer, including chronic inflammation associated with infections or autoimmune disease, inflammation caused by environmental and dietary exposure, tumor-associated inflammation, and therapy-induced inflammation (*39*). The targeting of inflammatory mediators (chemokines and cytokines, such as TNF-α and IL-1β), key transcription factors involved in inflammation (such as NF-κB and STAT3) or inflammatory cells decreases the incidence and spread of cancer (*40*). In various cancers, ICAM-

1 expression is at an elevated level and is associated with the malignant potential of cancer (*5-9*). To some extent, ICAM-1 overexpression is associated with the inflammatory environment in tumors. There is no report about the effect of mild hypothermia on cancer-related inflammation and the effect of mild hypothermia on the incidence and spread of cancer after radiotherapy, chemotherapy or surgery. In this study, we just investigated the effect of mild hypothermia on tumor cells adhesion to ECs *in vitro* and have not obtained information about the intricate systemic response *in vivo*. Therefore, it is worthwhile to find out whether mild hypothermia treatment will promote the outcome of animals with tumors after traditional therapy.

To investigate the potential mechanisms of the effect of mild hypothermia on tumor cells adhesion to ECs under our experimental conditions, tumor cell adhesion to immobilized ICAM-1 under flow at 35ºC and 37ºC was further conducted. The results showed that mild hypothermia could inhibit cells adhesion in flow when the density of adhesion molecules was kept constant. First, Rico and coworkers (*41*) observed that the work required to detach an atomic force microscope (AFM) cantilever tip functionalized with human ICAM-1-Fc from the cells' surface decreased dramatically from 37ºC to 16ºC. They further found it was due to two main factors. (1), reduced cell stiffness at higher temperatures would enhance the number of bonds formed by increasing the area of contact between the surfaces. (2), a lower linkage between the plasma membrane and cytoskeleton at higher temperatures would favor the extraction of long membrane tethers, which would prolong the time the cells remain in contact with the surface, favoring bond reformation. In addition, at the high experimental temperature, the membrane fluidity is increased and integrins realign themselves more effectively to bind with receptors (*42*). Second, it is worth mentioning the effect of temperature on functional conformation of adhesion molecules. It is well known that conformation of proteins is sensitive to temperature (*43*). Salas *et al*. (*44*) reported that LFA-1 (αLβ2) affinity for ICAM-1 was regulated by the conformation of the αL domain. The open or high affinity I domain conformation supports strong adhesion, whereas the closed, low affinity conformation mediates weak interactions or rolling. It was reported that LFA-1-dependent adhesion required divalent cations and was temperature-sensitive (*45,46*). Thus, it is likely that mild hypothermia may affect protein folding, which would be unfavorable to the formation of the high affinity conformation. Finally, decreased temperature may decrease the association rate constant of bonds, prolonging the time needed for cell attachment, as a consequence, cells detach from substrate before firm adhesion in the presence of shear force. The potential mechanisms of the effect of mild hypothermia on tumor cells adhesion to ECs should be further examined in detail, including the functional characteristics of ICAM-1.

Cell migration plays a central role in a wide variety of biological phenomena in both normal physiology and pathophysiology. Particularly in a tumor setting, cell migration is critical to metastasis. To test whether mild hypothermia could affect tumor cells migration, wound healing assays (non-oriented migration) and transwell migration assays (oriented migration) were performed. Because temperature affects cell proliferation (*47*), mitomycin C was used in the wound healing assay to avoid cell migration diversity due to cell proliferation differences affected by temperature. It is interesting that mild hypothermia can effectively weaken tumor cells oriented migration (chemotaxis to serum and SDF-1), but has no obvious effect on non-oriented migration. It is significant because a growing body of evidence shows that some tumor cells expressed chemokine receptors responding to chemokine gradients *in vitro*, and certain chemokines could serve as tissue-specific attractant molecules for tumor cells, promoting tumor-cell migration to particular sites *in vivo* (*48,49*). These results suggested that mild hypothermia may weaken chemokine-mediated metastatic spread of tumor cells, which is worthwhile to be investigated further.

In conclusion, results of the present study show that minimal mild hypothermia (35ºC) could reduce tumor cells adhesion to endothelium under physiological flow *in vitro* and weakened tumor cells oriented migration (chemotaxis). ICAM-1 was important for adhesion of human breast cancer cells MDA-MB-231 to ECs Eahy926, but mild hypothermia could not affect endothelial ICAM-1 expression. Reduced adhesion of tumor cells to endothelium may be caused by other mechanisms. These results suggest that mild hypothermia could be used as a potentially adjunct treatment combined with surgery to decrease tumor cell adhesion and migration. Further studies need to explore the accurate mechanism that mild hypothermia modulates adhesion and migration of tumor cells to endothelial cells.

Acknowledgements

This work was supported in part by grants from the National Natural Science Foundation of China (11172338, 11032012), the Program for New Century Excellent Talents in University (NCET-10-0879), the Natural Science Foundation Project of CQ CSTC (2009BB5043), the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry, the Fundamental Research Funds for the Central Universities (CDJZR 12238801, CDJXS 12230006), and the Sharing Fund of Chongqing University's Large-scale Equipment.

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(*Received July 1, 2012; Revised November 25, 2012; Accepted December 11, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.6.325

Pioglitazone attenuates myocardial ischemia-reperfusion injury *via* up-regulation of ERK and COX-2

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Summary **Our previous study demonstrated that the peroxisome proliferator-activated receptor (PPAR) γ agonist, pioglitazone (PIO), may be cardioprotective against ischemia-reperfusion injury; however, modulation of p42/p44 extracellular signal-regulated kinases (ERK1/2) and cyclooxygenase (COX)-2 by PIO in the myocardium with respect to ischemia-reperfusion (I/R) is only partially understood. We determined if PIO reduces I/R-induced apoptosis in cardiomyocytes, and whether or not this protective effect is due to modulation of ERK1/2 and COX-2. Sixty male Sprague-Dawley rats were randomized and assigned to 1 of 6 groups: I/R; I/R + PIO (5 mg•kg^{-1}•day^{-1}); I/R + PIO (10 mg•kg^{-1}•day^{-1}); I/R + PIO (10 mg•kg^{-1}•day^{-1}) + the ERK1/2 inhibitor, PD98059; I/R + PIO (10 mg•kg^{-1}•day^{-1}) + GW9662; and I/R + PD98059. Rats underwent 30 min of myocardial ischemia and 120 min of reperfusion, and then hearts were harvested for analysis. RT-PCR and Western blotting were performed to detect expression of ERK1/2 and COX-2. The number of TUNEL-positive cardiomyocytes and NEC in the PIO groups (5 and 10 mg•kg^{-1}•day^{-1}) was much lower than the I/R group. The cardioprotective effect of PIO was abrogated by PD98059 and GW9662. Phosphorylation of ERK1/2 and COX-2 was increased in the PIO-treated group compared with the I/R group. GW9662 reversed the expression of ERK1/2 and COX-2 phosphorylation induced by PIO. PD98059 reversed the expression of COX-2 induced by PIO. PIO was shown to be cardioprotective in an I/R injury model in rats *via* inhibition of cardiomyocyte apoptosis. PIO limited the infarct size in a PPAR-γ-dependent manner. These results show that PIO triggers the MAPK signaling pathway involving ERK1/2 using COX-2 as the downstream target.**

Keywords: Ischemia-reperfusion injury, apoptosis, peroxisome proliferator-activated receptor γ, pioglitazone, ERK1/2, COX-2

1. Introduction

Ischemia-reperfusion (I/R) injury is a major factor contributing to cardiac dysfunction and infarct size. In fact, I/R injury determines the prognosis after acute myocardial infarction. Cell death during I/R suggests two processes underlying the pathology of myocardial infarction (apoptosis and necrosis). Considerable evidence indicates that apoptosis is a significant contributor to myocardial cell death after I/R, particularly during the early stages (*1*). Studies suggest that targeting the reperfusion-induced apoptotic component of cell death can affect the apoptotic and necrotic components of cell death, the consequences of which are a reduction in infarct size and improved contractile function.

Our previous study demonstrated that the peroxisome proliferator-activated receptor (PPAR) γ agonist, pioglitazone (PIO), may protect the heart from I/R injury; the protective effect is likely to occur by inhibiting cardiomyocyte apoptosis (*1*). PPARs are nuclear hormone receptors that stimulate transcription of specific genes by binding to specific DNA

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sequences after activation by an appropriate ligand. Thiazolidinediones (TZDs: rosiglitazone, troglitazone, and PIO) are synthetic PPARγ ligands with well-recognized effects on glucose and lipid metabolism. It has recently become evident that the therapeutic effects of TZDs reach far beyond use as insulin sensitizers. Recently, several lines of evidence have suggested that TZDs protect the heart and other organs against tissue damage caused by I/R injury (*2*).

I/R has been shown to activate the pro-survival kinase signaling cascades, p42/p44 extracellular signal-regulated kinases (ERK1/2), which have been implicated in cell survival through recruitment of anti-apoptotic pathways of protection (*3*). The ERK1/2 signaling cascade is a mitogen-activated protein kinase (MAPK). The ERK1/2 signaling cascade is a family of serine-threonine kinases that are involved with regulation of the proliferation, differentiation, and survival of cells which are activated in response to the occupation of tyrosine kinase and G-protein-coupled receptors (*4*). The ERK1/2 cascade, when activated in the setting of I/R, can mediate cellular protection (*5,6*). Hausenloy *et al*. (*7*) demonstrated that ischemic pre-conditioning (IPC) protects the heart by phosphorylating the pro-survival kinases, ERK1/2, at reperfusion. The pharmacologic manipulation and up-regulation of pro-survival kinase cascades (the reperfusion injury salvage kinase (RISK) pathway) as an adjunct to reperfusion may therefore protect the myocardium from lethal reperfusion-induced cell death and provide a novel strategy to salvage viable myocardium and limit infarct size.

Data have shown that one of the downstream targets of ERK1/2 in IPC is cyclooxygenase (COX)-2 (*8*). COX-2 mediates the protective effects of ischemia-induced late pre-conditioning in rabbits and mice (*9*). Analyses of COX byproduct levels suggest that COX-2 mediates the late phase of cardioprotection *via* increased production of cytoprotective prostanoids (mainly prostaglandin (PG) I_2 and PGE_2) (*10,11*).

Ye *et al*. (*12*) reported that 3-day pre-treatment with pioglitazone (2.5 mg/kg/d) increased ERK1/2 phosphorylation in rat heart. Another TZD, rosiglitazone, augments ERK1/2 activation in myocardium of hypercholesterolemic rabbits subjected to I/R injury (*13*). Ye *et al*. (*14*) demonstrated that the infarct size-limiting effect of pioglitazone is COX-2-dependent. PIO, but not sitagliptin, increases cytosolic phospholipase A_2 and COX-2 activity in I/R models (*15*); however, modulation of COX-2 by the PPARγ agonist, PIO in the myocardium in relation to I/R has not been shown to be dependent on ERK.

We determined if PPAR-γ activation by PIO reduces I/R-induced apoptosis in cardiomyocytes and the area of necrosis in tissue, and if the underlying mechanism is related to modulation of the ERK1/2 and COX-2 pathways in the myocardium.

2. Materials and Methods

Experiments were carried out in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the Chinese PLA General Hospital (Beijing, China).

2.1. *Materials*

PIO was provided by Beijing Taiyang Pharmaceutical Industry Company, Ltd. (Beijing, China). Rabbit anti-ERK1/2 and anti-phosporylated ERK1/2 primary antibodies were purchased from Beijing Zhongshan Golden Bridge Biotechnology Company, Ltd. (Beijing, China). Rabbit anti-COX-2 primary antibody was purchased from Beijing Jingmei Biotech (Beijing, China). All other chemicals were reagent grade.

2.2. *Experimental preparation*

Sixty male Sprague-Dawley rats (190-240 g; Experimental Animal Center of the Academy of Military Medical Sciences, Beijing, China) were housed in a controlled environment at 25 ± 2°C with alternating 12 h light and dark cycles. All rats were acclimatized in our animal facility for ≥ 7 days before experiments. Stressful stimuli were avoided. Rats were randomized to 6 experimental groups of 10 rats each. Group 1 was the (I/R) group. Rats in group 1 were given 5% (v/v) dimethyl sulfoxide (DMSO, 10 $mL \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days before I/R. The second group was the I/R + PIO (5 $mg \cdot kg^{-1} \cdot day^{-1}$) group. Rats in group 2 were administered PIO (5 $mg \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days before I/R. The drug was initially dissolved in DMSO, then diluted in water, so that the final concentration of DMSO was < 5%, and adjusted to pH 7.4. After drug administration, rats had free access to standard rat food and water. The third group was the I/R + PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) group. Rats in group 3 were given PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days before I/R. Group 4 was the I/R + PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) + PD98059 group. Rats in group 4 were given PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days, then the ERK1/2 inhibitor, PD98059 (1 $mg \cdot kg^{-1}$, *iv*), 30 min before I/R. Group 5 was the I/R + PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) + GW9662 group. Rats in group 5 were given PIO (10 $mg \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days, then the selective PPARγ antagonist, GW9662 (1 $mg \cdot kg^{-1}$, *ip*) 30 min before I/R. Group 6 was the I/R + PD98059 group. Rats in group 6 were given 5% (v/v) DMSO (10 $mL \cdot kg^{-1} \cdot day^{-1}$) by gavage for 7 days, then the ERK1/2 inhibitor, PD98059 (1 $mg \cdot kg^{-1}$, *iv*) 30 min before I/R.

2.3. *Surgical procedures*

Rats were anesthetized with 10% urethane (10 $mL \cdot kg^{-1}$) before surgery. Anesthesia was maintained by supplementary injections of 10% urethane, as required.

The trachea was cannulated and the rats were ventilated with a ventilator (inspiratory oxygen concentration, 30%; 70 strokes•min^{-1}; tidal volume, 8-10 mL•kg^{-1}). A polyethylene catheter was inserted into the right carotid artery and connected to a pressure transducer to monitor blood pressure using an eight-channel polygraph recorder (Q1-160G; Nihon Kohden Corporation, Tokyo, Japan). Hemodynamic parameters were continuously monitored. The heart rate and mean blood pressure were noted at baseline (10 min after completion of surgery); at 10 min of ischemia; and at 10 min of reperfusion.

A snare occluder was placed around the left anterior descending artery (LAD). After completion of the surgical procedure, rats were allowed to stabilize for 30 min before LAD ligation. The coronary artery was occluded by tightening of the occluder. After 30 min of acute myocardial ischemia, the occluder was reopened to allow reperfusion for 120 min. The experiments were performed once.

After completion of the experiment, four hearts in each group were used to measure infarct size, and the other hearts were saved to determine apoptosis and expression of ERK1/2, pERK1/2, and COX-2. The number of hearts was 5, 5, 4, 6, 6, and 6 in groups 1-6, respectively.

2.4. *Quantification of injury to myocardial tissue*

At the end of the 120 min reperfusion period, the LAD was re-occluded and 1 mL of Evans blue dye (2% w/v) was injected into the right carotid artery. The Evans blue dye stained the tissue through which it could circulate, so that non-perfused vascular (occluded) tissue remained colorless. The rats were sacrificed by decapitation after aortic exsanguination. The heart was excised, and excess dye was washed off.

The heart was sectioned into slices 3-4 mm in thickness, and the wall of the right ventricle was removed. The ischemic area (area at risk (AAR)) was distinguished from the area not at risk by Evans blue dye staining. The infarcted portion of the myocardium (necrotic area (NEC)) was determined using the triphenyl tetrazolium chloride (TTC) method. The two portions (AAR and NEC) of the left ventricle were quantified by use of Image-Pro software.

2.5. *Preparation for histologic examination*

The hearts were quickly sectioned at the end of reperfusion. The I/R tissue was separated, fixed in 10% neutral formalin in phosphate-buffered saline (PBS; pH 7.4), and embedded in paraffin wax. The I/R tissue was then cut into 4 μm-thick sections for terminal dUTP deoxynucleotidyltransferase nick end-labeling (TUNEL) assay after deparaffinization and rehydration.

2.6. *Detection of in-situ cell death*

Apoptotic cells were identified using an *In Situ* Cell Death Detection Kit POD (Roche Applied Science, Mannheim, Germany). The kit permits immunohistochemical detection and quantification of apoptosis at the level of a single cell based on labeling of DNA strand breaks. Tissue sections were incubated with freshly prepared 3% hydrogen peroxide solution for 10 min at room temperature to block endogenous production of peroxidase, then with terminal deoxynucleotidyl transferase for 1 h at 37°C. Total and TUNEL-positive cardiomyocytes were counted in five random visual fields per tissue section using image analysis software. The results are expressed as the apoptosis index, as follows: apoptosis index = ((number of TUNEL-positive cardiomyocytes/total cardiomyocytes) ×100).

2.7. *Reverse transcription-polymersase chain reaction (RT-PCR) for COX-2*

Heart tissues were removed from the thoracic cavity, immediately frozen in liquid nitrogen, and stored at –70°C until RNA extraction. Total RNA was extracted from tissues with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 2 g of RNA was treated with ribonuclease-free deoxyribonuclease, and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Two microliters of synthesized cDNA were subjected to 30 cycles of PCR, which resulted in a single specific amplification product of expected size. The PCR conditions were as follows: 45 sec denaturation at 94°C; 45 sec annealing at 60°C (COX-2, β-actin); and 45 sec extension at 72°C. The PCR primers used were as follows: COX-2 sense, 5′-AGTATCAG AACCGCATTGCC-3′ and antisense, 5′-TAAGGTTTCAGGGAGAAGCG-3′; and β-actin sense, 5′-AAGTACCCCATTGAACACGG-3′ and antisense, 5′-ATCACAATGCCAGTGGTACG-3′. β-Actin was used as an internal control for the PCR. The cycle numbers were 30 (COX-2) and 26 (β-actin). The RT-PCRs were in the linear range of amplification for the target mRNA, as well as for the control. Each RT-PCR product was revealed on 1% agarose gels stained with ethidium bromide. The bands were documented, scanned, and quantified using Quantity One software (PDI, New York City, NY, USA) and normalized with the internal control (β-actin). Three repeated tests were performed for each set of measurements and the resulting data were averaged. The results are expressed as the fold change over the I/R group.

2.8. *Western blot analyses for ERK1/2, pERK1/2, and COX-2*

Rat heart tissues were washed twice in ice-cold PBS.

Cytosolic extracts were prepared by homogenizing the tissues in an extraction buffer with freshly added 1 mM Na_3VO_4, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 g/mL leupeptin, and 2 g/mL aprotinin. Protein concentrations were determined using the bicinchoninic acid assay. Approximately 15 μg of total protein was loaded. Proteins were separated on 10% or 12% sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Blots were blocked in blocking buffer containing PBS with 5% albumin. Membranes were incubated with primary antibody (rabbit anti-ERK1/2, rabbit anti-phosphorylated ERK1/2, and rabbit anti-COX-2). Blots were then incubated with secondary antibody conjugated with horseradish peroxidase (1:10,000) for 30 min at room temperature. Blots were developed with an enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK). Three repeated tests were performed for each set of measurements and the resulting data were averaged. The results are expressed as the fold change over the I/R group.

2.9. *Statistical analyses*

Data are the mean ± standard error (S.E.) of *n* observations, where *n* represents the number of rats in the group. For multiple group comparison, data were analyzed by one-way ANOVA, followed by the Student's *t*-test for comparison between two groups. A $p < 0.05$ was considered significant.

3. Results

Sixty rats were included in the protocol. Overall, there were nine rats in the I/R group (one rat was excluded because of a lack of ischemia). There were 9 rats in the PIO 5 mg•kg^{-1} group (1 died during ischemia). There were 8 rats in the PIO 10 mg•kg^{-1} group (2 rats died during reperfusion). None of the rats were excluded from the other groups.

There were no differences in body weight among the groups (Table 1). Heart rate and mean blood pressure are presented in Tables 2 and 3. Overall, there were no statistically significant differences among groups with respect to heart rate and mean blood pressure.

3.1. *PIO decreased the size of myocardial necrosis induced by I/R injury in rat hearts*

The mean values for the AAR, determined by Evans blue dye after reperfusion and expressed as percentages of LV (AAR/LV), showed no difference among the groups (Figure 1). NEC/left ventricle was significantly reduced in the PIO 5 mg•kg^{-1} (by 20%) and PIO 10 mg•kg^{-1} (by 23%) groups compared with the I/R group. NEC/left ventricle was significantly increased in the PIO 10 mg•kg^{-1} + PD98059 group (by 12% and 15%) compared with the PIO 5 mg•kg^{-1} and PIO 10 mg•kg^{-1} groups, respectively. NEC/left ventricle was significantly increased in the PIO 10 mg•kg^{-1} + GW9662 group (by 17% and 20%) compared with the PIO 5 mg•kg^{-1} and PIO 10 mg•kg^{-1} groups, respectively. There were no significant differences between the I/R

Table 1. Body weights

Items	I/R	PIO (5)	PIO (10)	PIO (10) + PD	PIO (10) + GW9662	PD	*p* value
n	9	9	8	10	10	10	
Body weights	228 ± 6	219 ± 7	231 ± 6	214 ± 8	223 ± 5	216 ± 6	0.4069

Values are the mean ± S.E.; *n*, no. of rats.

Table 2. Average heart rate during IS experiments

Items	I/R	PIO (5)	PIO (10)	PIO (10) + PD	PIO (10) + GW9662	PD	*p* value
n	9	9	8	10	10	10	
Baseline	207 ± 4	202 ± 6	209 ± 6	208 ± 5	210 ± 7	207 ± 4	0.9311
10-Min occlusion	212 ± 4	205 ± 5	213 ± 4	208 ± 7	209 ± 5	209 ± 5	0.9094
10-Min reperfusion	211 ± 6	203 ± 6	212 ± 4	206 ± 7	207 ± 3	206 ± 4	0.8482

Values (in beats/min) are the mean ± S.E. heart rate. At baseline, during coronary occlusion and reperfusion, there were no differences among the groups.

Table 3. Mean blood pressure during IS experiments

Items	I/R	PIO (5)	PIO (10)	PIO (10) + PD	PIO (10) + GW9662	PD	*p* value
n	9	9	8	10	10	10	
Baseline	108 ± 4	113 ± 3	109 ± 7	110 ± 5	105 ± 5	109 ± 4	0.9156
10-Min occlusion	106 ± 3	109 ± 5	108 ± 7	108 ± 3	102 ± 4	104 ± 5	0.8863
10-Min reperfusion	100 ± 6	105 ± 4	106 ± 4	105 ± 5	99 ± 3	100 ± 4	0.7735

Values (in mmHg) are the mean ± S.E. blood pressure (MBP). At baseline, during coronary occlusion and reperfusion, there were no differences among the groups.

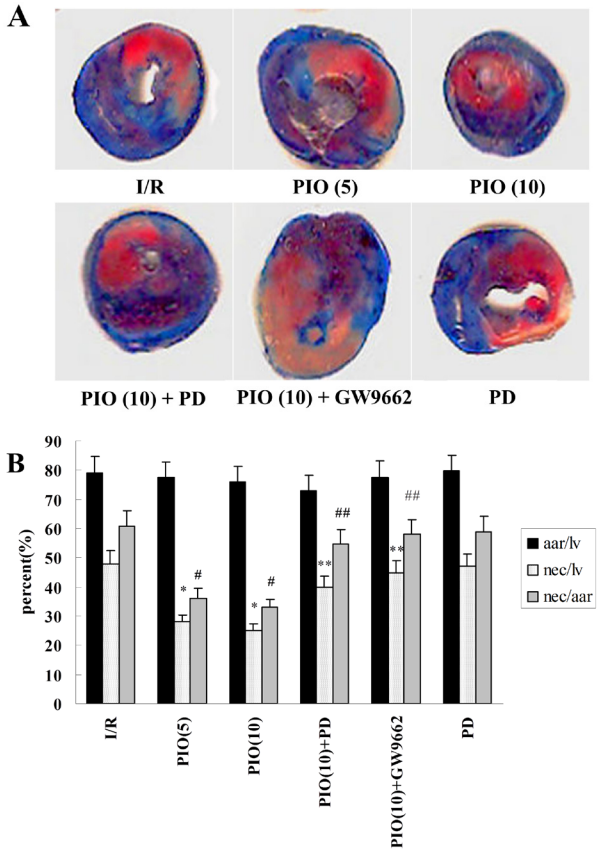

Figure 1. Effects of pioglitazone on ischemia–reperfusion injury. (A), Representative heart sections from I/R and PIO groups with the ischemic area consisting of both the red and pale regions. The ischemic area was determined by negative stain with Evans blue and the infarct area was detected by TTC staining as described in methods section . Note that the area of infarction is the pale zone, indicative of dead tissue. The area not at risk is stained blue. (**B)**, Effects of pioglitazone on MI size..Results are the mean ± S.E. #, * $p <$ 0.05 *vs*. I/R group, ##, ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups. aar: areas at risk; nec: area of necrosis.

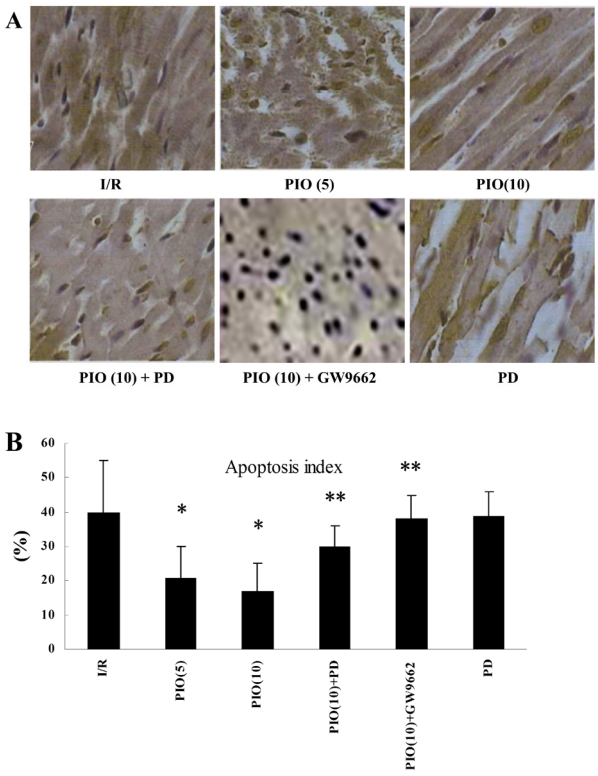

Figure 2. Effect of pioglitazone on cardiomyocyte apoptosis in the ischemia–reperfusion model of rat hearts analyzed by the TUNEL assay (×400). Representative TUNEL showed apoptotic cardiomyocytes from rats along with statistic analysis of apoptotic index. Apoptotic cells were determined using an *In Situ* Cell Death Detection Kit POD. PIO reduced the number of apoptotic cardiomyocytes compared with the ischemia-reperfusion group. Results are the mean ± S.E. * $p < 0.05$ *vs*. I/R group; ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups.

and PD98059 groups, indicating that PD98059 did not affect infarct size when given alone. NEC/AAR was noticeably reduced in the PIO 5 mg•kg^{-1} (by 24%) and 10 mg•kg^{-1} (by 28%) groups compared with the I/R group. NEC/AAR was significantly increased when PIO was used along with PD98059 and GW9662. There was no significant difference in NEC/left ventricle and NEC/AAR between the two PIO subgroups.

3.2. *PIO reduced the myocardial apoptosis index*

In the PIO 5 mg•kg^{-1} and PIO 10 mg•kg^{-1} groups, the number of TUNEL-positive cardiomyocytes was much lower than the I/R group (Figure 2). The apoptosis index was significantly increased when PIO was used along with PD98059 and GW9662. There were no significant differences between the I/R and PD98059 groups.

3.3. *PIO activated ERK1/2 signaling pathways*

Pre-treatment of rat hearts with PIO (5 and 10 mg•kg^{-1}) significantly enhanced the phosphorylation of ERK1/2 compared with the I/R group. The ERK1/2 levels were not significantly different among the groups. The PIO-mediated increase in phosphorylation of ERK1/2 (Figure 3) was reduced by PD98059 and GW9662.

3.4. *PIO up-regulated COX-2 expression in I/R myocardium*

COX-2 levels were significantly increased by pre-treatment with PIO (5 and 10 mg•kg^{-1}) at the mRNA and protein levels compared with the I/R group (Figures 4 and 5). The increased levels of COX-2 were significantly reduced by PD98059 and GW9662.

4. Discussion

We report here that pre-treatment with PIO for 7 days caused a substantial reduction in the apoptosis index and infarct size in rats undergoing regional myocardial I/R. Furthermore, these beneficial changes were accompanied by activation of the ERK1/2 pathway and increased expression of COX-2 at the mRNA and protein levels, which was blocked by GW9662, the

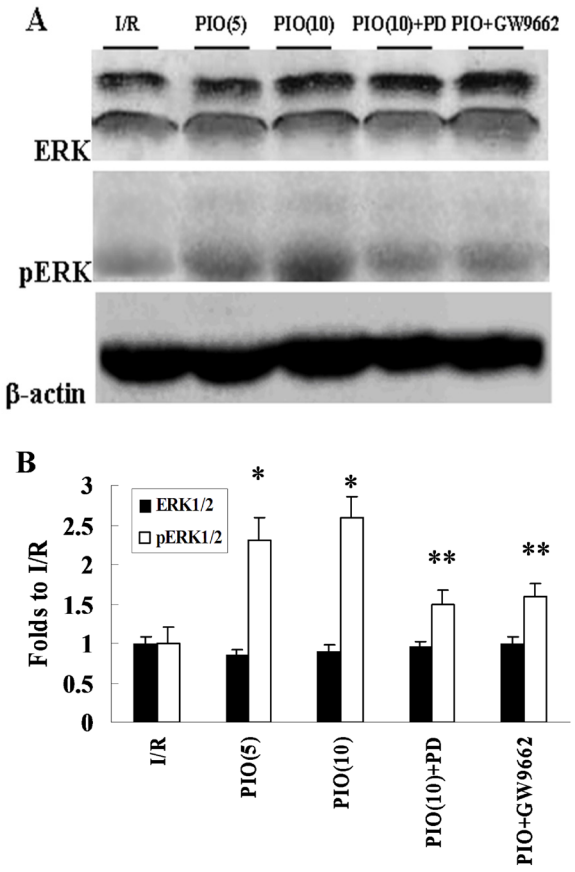

Figure 3. The WB analysis was performed on individual samples of each group. Representative immunoblot **(A)** and densitometric analysis **(B)** of ERK expression. Data are fold change compared with expression in the I/R group. Overall, there were significant differences among groups ($P < 0.05$). Pre-treatment with PIO (5 and 10 mg•kg^{-1}) significantly enhanced the phosphorylation of ERK compared with the I/R group. PIO-mediated increased phosphorylation of ERK1/2 was reduced by PD98059 and GW9662. * $p < 0.05$ *vs*. I/R; ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups.

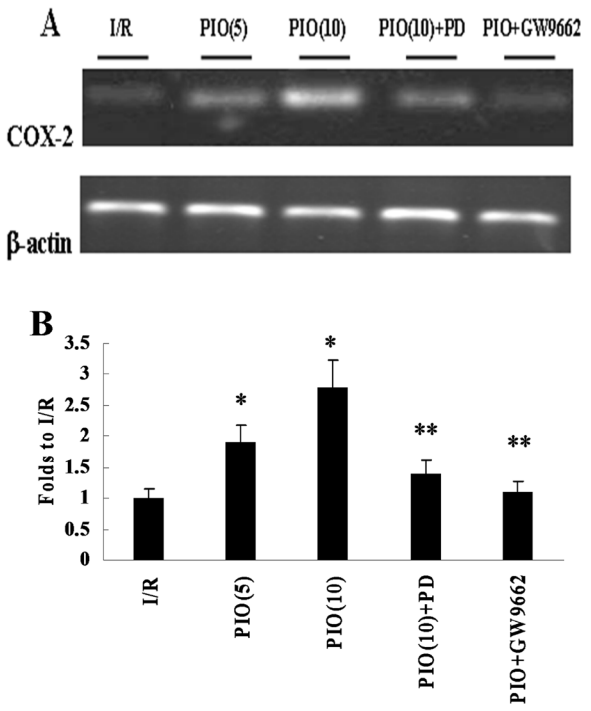

Figure 4. Effects of pre-treatment with pioglitazone on COX-2 mRNA expression. The RT-PCR analysis was performed on individual samples of each group. Relative densitometric units of COX-2/β-actin are shown in the histogram, with the density of the I/R bands set at 1.0. * $p < 0.05$ *vs*. I/R; ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups.

selective PPARγ antagonist. Pre-treatment with the ERK1/2 inhibitor, PD98059, partially blocked the effects exerted by PIO. Increased levels of COX-2 by PIO were significantly reduced by PD98059.

Myocardial ischemia-reperfusion is a problem associated with re-establishment of blood flow in coronary bypass surgery, thrombolysis, and angioplasty. Evidence indicates that apoptosis is a significant contributor to myocardial cell death after I/R. Studies have demonstrated that the pharmacologic inhibition of the apoptotic signaling cascade can attenuate the apoptotic and necrotic components of cell death (*16,17*). In addition to the apoptotic component of cell death contributing to the extension of infarct size during reperfusion, Zhao *et al*. (*18*) demonstrated that pharmacologic inhibition of the reperfusion-induced apoptotic component of cell death also resulted in improved contractile function of ischemic canine hearts. In healthy, diabetic, or obese animals, PPARγ agonists reduced the size of myocardial infarcts (*19*). These effects are associated with increased uptake of glucose

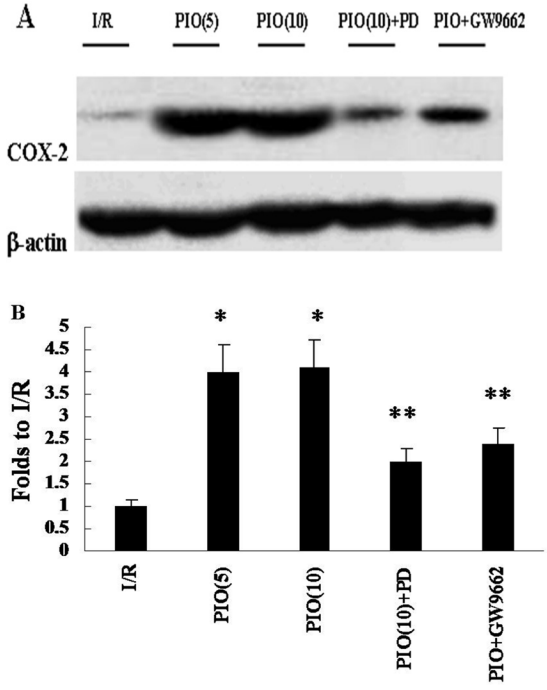

Figure 5. Effects of pre-treatment with pioglitazone on COX-2 protein expression. Each immunoblot is from a single experiment and is representative of all separate experiments. Relative densitometric units of COX-2/β-actin are shown in the histogram, with the density of the I/R bands set at 1.0. Densitometry results are the mean ± S.E. of individual experiments. * $p < 0.05$ *vs*. I/R; ** $p < 0.05$ *vs*. PIO (5 and 10 mg•kg^{-1}) groups.

and improved sensitivity to insulin. PPARγ agonists also reduce post-ischemic myocardial apoptosis (*13*). One study demonstrated the neuroprotective activity of PIO in global cerebral I/R injury and attributed the neuroprotective effects to a reduction in oxidative stress and DNA fragmentation (*20*). Based on the available evidence, the signaling molecules involved in the protection of PPAR-γ agonists include ERK (*12,13*), COX-2 (*14,15*), phosphatidylinositol-3 kinase (PI3K)/Akt, and microRNA-29. The PI3K pathway plays an important role in regulating numerous biological processes, including survival, proliferation, adhesion, migration, insulin activity, and cell activation. The protection of PIO against I/R injury is abolished by PI3K inhibitors (LY294002 or wortmannin); thus, the protection of PIO appears to involve PI3K-Akt (*21*). Nevertheless, there are conflicting reports regarding the effect of TZDs on Akt phosphorylation. In another study, PIO caused a minor, but insignificant increase in myocardial P-Akt expression (*14*). This suggests that the IS-limiting effect of PIO is probably independent of Akt phosphorylation. Down-regulation of miR-29 by an antisense inhibitor or by PIO protects H9c2 cells from simulated I/R injury, as indicated by increased cell survival and decreased caspase-3 activity (*22*); however, it has not been clarified that modulation of COX-2 by the PPARγ agonist, PIO, in the myocardium in relation to I/R is dependent on ERK.

In the present study, pre-treatment with the PPARγ agonist, PIO, markedly increased the level of phosphorylated ERK1/2 in rat hearts. PD98059 reversed the cardioprotection exerted by PIO. Increased levels of COX-2 by PIO were significantly reduced by PD98059. These results imply that PIO decreases apoptosis induced by I/R injury through the ERK1/2 signaling pathway using COX-2 as the downstream target.

The role of ERK1/2 activation in mediating TZD-induced cardioprotection is not clear. Administration of U0126 or PD98059, both of which are P42/44 MAPK inhibitors, before ischemia did not attenuate the infarct size-limiting effects of pioglitazone in Langendorff perfused rat hearts. In contrast, when administered just before reperfusion, both inhibitors blocked the protective effects of PIO, suggesting that ERK1/2 activation only affects reperfusion injury (*21*).

Activation of the ERK1/2 cascade would be expected to reduce cell death through several anti-apoptotic mechanisms (*23*). Based on the available evidence, it appears that BAD, BAX, p70S6K, and eNOS appear to be the downstream components responsible for mediating the protection associated with activation of the ERK1/2 cascade at the time of reperfusion. Data have shown that cardiac damage caused by oxidative stress after I/R was limited by ERK1/2-mediated induction of COX-2 (*8*). The present study confirmed that PIO increased the expression of COX-2 at the mRNA and protein levels. Increased expression of COX-2 was significantly reduced by PD98059, thus COX-2 may be downstream of ERK1/2. Recent studies have demonstrated that IPC up-regulates the expression and activity of COX-2 in the heart, and this increase in COX-2 activity mediates the protective effects of the late phase of IPC against myocardial stunning and infarction. Shibata *et al*. (*24*) reported that COX-2 mediated the cardioprotection from I/R injury due to adiponectin. Neilan *et al*. (*25*) reported that genetic disruption of COX-2 increases cardiac dysfunction after treatment with doxorubicin due to an increase in apoptosis of cardiac cells, suggesting that COX-2 and prostacyclin modulate the expression of genes encoding for proteins involved in apoptosis. Ye *et al*. (*26*) reported that the myocardial protective effect of PIO is iNOS-independent and may be only partially dependent on eNOS. Up-regulation of COX-2 by PIO is independent of NOS (*26*).

In the present study, the selective PPARγ antagonist, GW9662, abolished the protective effects of PIO, suggesting that the protective effects of PIO is likely PPARγ-dependent.

In general, pre-treatment with PIO in rats may protect the myocardium from I/R injury, as demonstrated by the reduction in the apoptosis index and size of the myocardial infarct. PIO might exert protection against I/R injury through the ERK1/2 pathway using COX-2 as the downstream target.

Acknowledgements

This work was supported by a grant from the National Nature Science Foundation of China (grant number 81270941).

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(*Received June 3, 2012; Revised December 12, 2012; Accepted December 25, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.6.333

An eligible biological allograft patch in tension-free herniorrhaphy of swine

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Summary **Current patches made from macromolecular compounds or composix for tension-free herniorrhaphy are still unsatisfactory in biocompatibility. The ideal patch should be a biological patch with good biocompatibility. Herein allograft patches modified by tissue engineering were used in tension-free herniorrhaphy of swines. Tough membrane tissues from swine were modified with patented tissue engineering techniques to develop allograft patches for tension-free herniorrhaphy. Histological, and physical tests of the allograft patch were performed subsequently, which revealed that the allograft patch was sufficient and satisfactory for tension-free herniorrhaphy. The allograft patches were next used in tension-free herniorrhaphy of abdominal external hernia models of swines and compared to polypropylene patches. Serous $CD4^+$, $CD8^+$ T cells, interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α) were determined preoperatively and postoperatively. Local pathological changes were recorded postoperatively in swines. *In vivo* application of the allograft patches revealed that there were no significant serous cellular immune responses in swines, and inflammation induced by allograft patches was significantly lower compared to polypropylene patches, the allograft patches gradually degenerated and new collagen fibers appeared. Abdominal external hernias were cured with allograft patches and without relapse. The modified allograft patch with satisfactory biocompatibility was eligible and sufficient in tension-free herniorrhaphy of swine. Clinical trials should be performed for further evaluation of the allograft patch.**

Keywords: Biological allograft patch, tissue engineering modification, tension-free herniorrhaphy, swine

1. Introduction

External abdominal hernia is a frequently occurring disease that severely impairs quality of life and may sometimes be fatal. It is estimated that 1.5% of all people suffer from this disease. Worldwide, over 1 million external abdominal hernia surgeries are performed annually. The vast majority (90%) of external abdominal hernias are inguinal hernias and, in fact, 25% of males and 2% of females will develop an inguinal hernia during their lifetime, resulting in a high prevalence of external abdominal hernias (*1*). Roughly 3-20% of the patients undergoing open abdominal operations suffer from incisional hernias (*2*).

Surgical treatment of tensional herniorrhaphy revealed several shortcomings, and tension-free herniorrhaphy was first proposed by an American surgeon, Lichtenstein, in 1898 (*3*). Artificial patches were used for herniorrhaphy, which can minimize relapses and complications. There are two kinds of patches: artificial patches made from macromolecular compounds and biological patches (*4-6*). The most common macromolecular materials used for patches are polypropylene and polytetrafluoroethylene (*7-9*); however, there are several shortcomings of artificial

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macromolecular patches, *e.g*. chronic and long-term pain, foreign body sensation, patch translocation to other organs, formation of fibrous capsules, intestinal obstruction and relapse (*10-12*).

Researches have been focused on the use of composix mesh (*13-15*) or natural biological materials based on animal tissues (*16,17*); however, these materials are relatively unstable, degrade easily, and have poor mechanical strength. Furthermore, animal tissues may induce immunological rejection, thus adversely affecting their application (*18*).

Herein tough membrane tissues from swine were modified with tissue engineering techniques to develop allograft patches in tension-free herniorrhaphy of swines.

2. Materials and Methods

2.1. *Generation of the biological allograft patch*

Tough membrane materials from swines (Animal experiment center of Grandhope Biotech Co., Ltd. Guangzhou, China) were processed by a series of patented tissue engineering techniques (Patent Number: US 6, 101, 555 and US 6, 231, 614B1). Animal use was approved by the Animal Ethics Committee of Guandong province. These techniques included fixation of animal tissues, removal of antigenic epitopes, induction of tissue growth, modification of proteins and adjustment of degradation for the biological materials. Pericardium from fresh swines were collected, pretreated and fixed with an epoxide crosslinker, foreign antigens were removed and proteins were modified. After optimization of the physical strength, cutting, packaging and sterilization processes, the patch was developed.

2.2. *Morphological observation, and determination of physical parameters of the biological allograft patch*

Hematoxylin and Eosin (HE; IHC World, Woodstock, MD, USA) staining were performed on the biological allograft patch according to the manufacturer's instructions, and subsequently observed under a light microscope (Olympus, Tokyo, Japan). The biological allograft patch was also prepared for observation under an H-600 transmission electron microscope (Hitachi, Tokyo, Japan) for ultra microstructure.

Tensile strength and stretch rate of the biological allograft patch was determined with a micro-control electronic tensile testing machine (JPL, Jiangshu, China), and the sample was prepared and tested according to the manufacturer's instructions. Values were presented as N/cm and %.

2.3. *Tension-free herniorrhaphy to external abdominal hernia models of swines with allograft patches*

18 female swines (weight: 20-25 kg) were obtained from the animal experiment center of Grandhope Biotech Co., Ltd., Guangzhou, China. Animal use was approved by the Animal Ethics Committee of Guandong province. All swines were anesthetized 30 min before operations with ketamine hydrochloride injection (Shanghai No.1 Biochemical & Pharmaceutical Co., Ltd., Shanghai, China) by intramuscular injection (0.28 mL/kg). A 10 cm incision in the upper abdomen was made vertically through the linea alba to the parietal peritoneum in order to produce an external abdominal hernia.

All 18 female swines presenting with successful external abdominal hernias were equally and randomly assigned to 2 groups for tension-free herniorrhaphy 2 weeks after the first operation for the external abdominal hernia model, one group with allograft patches (n = 9), while the other group with polypropylene patches (C.R. Bard, Murray Hill, NJ, USA) (n = 9). The original incision was cut and the hernia sack was exposed. Tension-free herniorrhaphy was performed to repair the hernia. Swines were injected postoperatively and intramuscularly with penicillin at 50,000 IU/kg, twice a day (Baiyunshan Tianxin pharmaceutical Co., Ltd., Guangzhou, China) for 3 continuous days to prevent infection. 6 months after herniorrhaphy, 2 swines from each group were sacrificed for observation of the implanted patches, the other 14 swines were observed for 12 months after herniorrhaphy. All the implanted patches and surrounding tissues were observed under a light microscope (Olympus) and an H-600 transmission electron microscope (Hitachi).

2.4. *Serous $CD4^+$, $CD8^+$ T cells, interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α) of swines*

Serum samples were collected from all swines before herniorrhaphy, 1 week and 4 weeks after herniorrhaphy for determination of $CD4^+$, $CD8^+$ T cells, IL-1β, and TNF-α. Briefly, three-color flow cytometry was employed using an Enzymatic Amplification Staining Kit (Flow-Amp Systems, Tebu-bio, Le Perray en Yvelines, France) for proportions of $CD4^+$, and $CD8^+$ T cells. Specific anti-swine antibodies: anti-CD4-FITC, anti-CD8-FITC (Southern Biotechnology Associates Inc., Birmingham, UK) were used according to the manufacturer's instructions. An enzyme-linked immunosorbent assay was used to detect the concentration of IL-1β and TNF-α in the serum, rabbit monoclonal anti-IL-1β and anti-TNF-α (Becton Dickinson, Franklin Lakes, NJ, USA) were used as the primary antibodies.

2.5. *Statistics*

Data are presented as the means ± S.D. Student's t-tests were used for statistical analyses. SPSS 11.5 for

windows was used. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Morphology and physical parameters of the biological allograft patch*

The biological allograft patches displayed a milky white color. One side of the patch was smooth while the other side had faint striated structures. Patches contained small, evenly distributed holes (Figure 1A). The maximum patch size was 21 × 16 cm with a thickness of 0.3-0.7 mm. The patches were fairly flexible and plastic, and were easily sutured and tailored.

HE staining showed that the patch was composed of regularly-aligned collagen, and displayed no evidence of cells or vessels (Figure 1B). Transmission electron microscopy revealed that the collagen was aligned regularly and inseparably. Cells and vessels were not seen (Figure 1C).

The physical parameter of tensile strength of the biological allograft patch was 90-160 N/cm (median 125 N/cm), and the stretch rate of the biological patch was 62-82% (median 71%).

3.2. *External abdominal hernia models and tension-free herniorrhaphy with allograft patches*

All swines survived the operation of the external abdominal hernia model. In the operation, muscle laying on both sides of the linea alba was separated and the enclosed muscle fiber was transected, leading to the formation of an 8 × 6 cm defect area (Figure 1D), 2 days later, the incisional sites began to protrude, and 7 days after operation, a classical external abdominal hernia had formed (Figures 1E and F). The size of the hernia was 12 × 10 cm, projecting 4 cm over the skin when the swine was in the court position.

There were no infections or impaired wound healing for both groups that underwent tension-free herniorrhaphy with allograft or polypropylene patches. In tension-free herniorrhaphy with allograft patches, the allograft patch was placed onto the defect area, and overlaps between the patch and hernia had to be larger than 1 cm (Figures 1G and H). All external abdominal

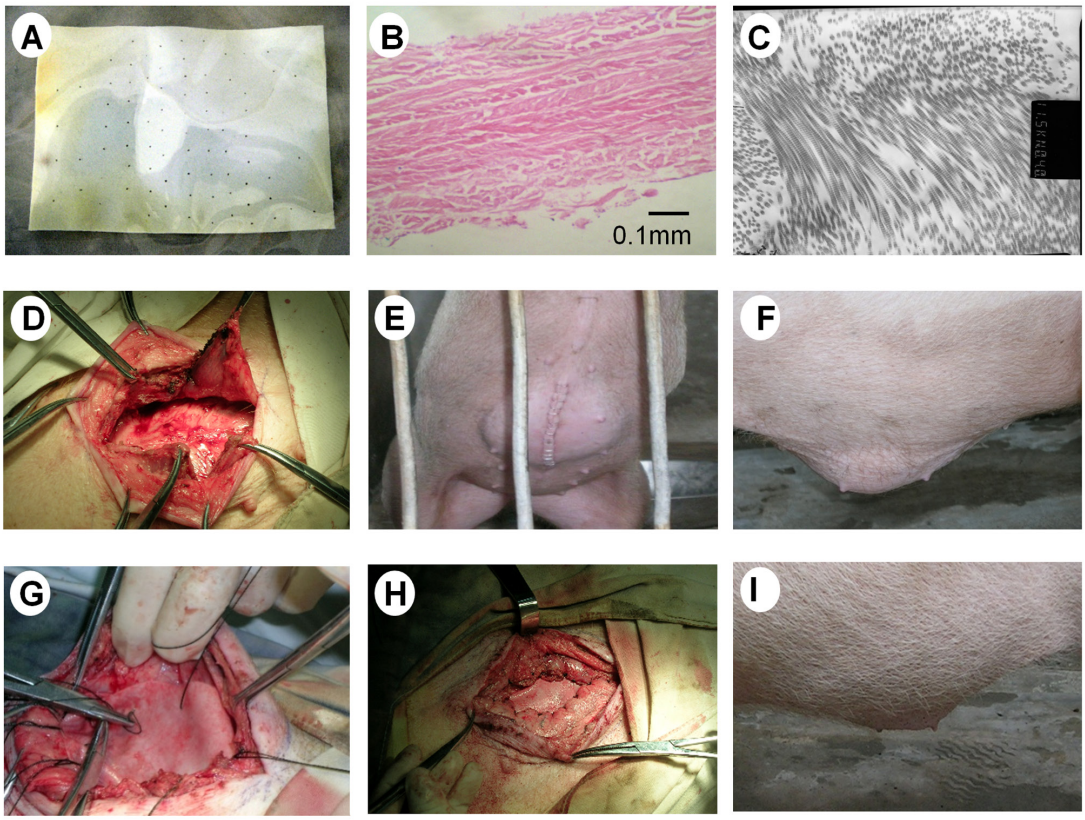

Figure 1. Histological observation of the biological allograft patch and its subsequent application in tension-free herniorrhaphy to external abdominal hernia models of swines. The biological allograft patch that has small, evenly-distributed holes **(A)**; Regularly-aligned collagen was observed and cells were not observed in the patch according to HE staining **(B)**; Collagen was aligned regularly and inseparably, cells and vessels were not observed according to transmission electron microscopy **(C)**; In operation of model of external abdominal hernia, muscle laying on both sides of the linea alba was separated and the enclosed muscle fiber was transected, leading to the formation of a 8 × 6 cm defect area **(D)**; A classical external abdominal hernia had formed 7 days after the operation of model **(E, F)**; In tension-free herniorrhaphy, process of suturing the patch with the swine tissues **(G)**; Observations after the allograft patch was implanted onto the defect area, the overlaps between the patch and hernia were larger than 1 cm **(H)**; The external abdominal hernia was cured without relapse 12 months after herniorrhaphy **(I)**.

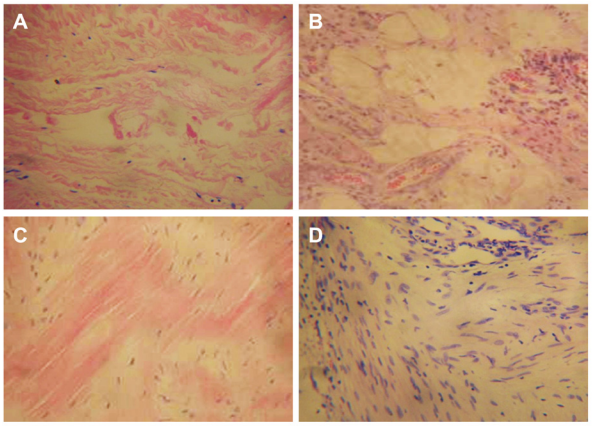

Figure 2. Histological observation under light microscopy of the allograft and polypropylene patches 6 and 12 months post-herniorrhaphy. Degenerated and broken collagen fibers appeared, and many fibroblasts and fibrocytes were observed in allograft patches 6 months after herniorrhaphy (×200) **(A)**; Megakaryocytes and fibrocytes among polypropylene patches were observed and collagen fibers appeared irregularly 6 months after herniorrhaphy (×200) **(B)**; New regular collagen fibers were observed among the allograft patch 12 months after herniorrhaphy (×200) **(C)**; Megakaryocytes and irregular new collagen fibers were observed among polypropylene patches 12 months after herniorrhaphy (×200) **(D)**.

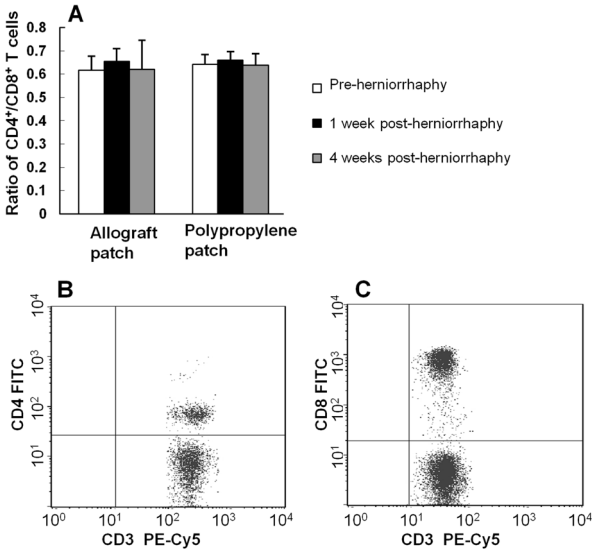

Figure 3. Serous ratios of $CD4^+/CD8^+$ T cells of swines in both groups. There were no significant differences of serous ratios of $CD4^+/CD8^+$ T cells between groups of allograft and polypropylene patches before herniorrhaphy, 1 week and 4 weeks after herniorrhaphy **(A)**; Proportions of serous $CD4^+$ and $CD8^+$ T cells in one swine 4 weeks after herniorrhaphy were 23.22% **(B)** and 34.18% **(C)**, respectively.

hernias of swines were cured without relapses within 12 months observation after herniorrhaphy (Figure 1I).

3.3. *Histological analysis of implanted allograft patches 6 months and 12 months after tension-free herniorrhaphy*

Six months after herniorrhaphy, degenerated and broken collagen fibers appeared, and many fibroblasts and fibrocytes were observed in allograft patches under light microscopy (Figure 2A). The allograft patches were surrounded by a large number of fibroblasts, eosinophils, lymphocytes and continuous wave-like collagens, which were confirmed with transmission electron microscopy. Light microscopy for polypropylene patches revealed that there were lots of megakaryocytes and fibrocytes among polypropylene patches, collagen fibers appeared irregularly (Figure 2B).

Twelve months after herniorrhaphy, there were new collagen fibers appearing and presenting regularly, and a few sporadic fibroblasts among the allograft patches (Figure 2C). Results of polypropylene patches revealed that there were lots of megakaryocytes among polypropylene patches and new collagen fibers appearing irregularly (Figure 2D).

3.4. *Determination of serous $CD4^+$, $CD8^+$ T cells, IL-1β, and TNF-α of swines*

The proportions of $CD4^+$ cells and ratios of $CD4^+/CD8^+$ T cells of swines between groups of allograft and polypropylene patches were not significantly different pre-herniorrhaphy, 1 week and 4 weeks post-herniorrhaphy (Figure 3). In the group of allograft patches, the proportions of $CD4^+$ cells and ratios of $CD4^+/CD8^+$ T cells of swines were not significantly different between pre-herniorrhaphy, 1 week and 4 weeks post-herniorrhaphy (Table 1). ELISA results revealed that serous IL-1β (Table 2) and TNF-α (Table 3) increased 1 week post-herniorrhaphy, and decreased 4 weeks post-herniorrhaphy but remained higher than that of pre-herniorrhaphy. Results of the IL-1β and TNF-α group of polypropylene patches were significantly higher than that of the group of allograft patches.

4. Discussion

Studies on scaffold materials are one of the most important research areas in tissue engineering. Not only can scaffold materials fix structural tissue damage, but they can also provide a three-dimensional vector with which functional cells can anchor, grow and proliferate. As the scaffold materials are degraded and absorbed, gradually new, regenerated tissue begins to replace the old and diseased tissue leading to functional improvement of the pathological tissue or organ (*19*). Materials for external abdominal hernia repair also belong to the category of scaffold material research areas (*20*).

Lately, research on biological patches has been focused on the use of animal tissue-based natural materials (*21*).

Classical methods for treating the animal tissue are based on using glutaraldehyde for fixation and

Table 1. Proportions (%) of $CD4^+$ T cells and ratios of $CD4^+/CD8^+$ T cells of swines in the group of allograft patches

Items	pre-herniorrhaphy	1 week post-herniorrhaphy	4 weeks post-herniorrhaphy
Proportions (%) of $CD4^+$ T cells	21.89 ± 2.24	23.11 ± 1.94	21.93 ± 2.24
Ratios of $CD4^+/CD8^+$ T cells	0.618 ± 0.059	0.656 ± 0.054	0.621 ± 0.124

$p > 0.05$

Table 2. Serous IL-1β of swines in both groups (pg/mL)

Items	pre-herniorrhaphy	1 week post-herniorrhaphy	4 weeks post-herniorrhaphy
Group of allograft patch	8.98 ± 5.79	202.31 ± 17.41*	38.60 ± 25.87**
Group of polypropylene patch	10.13 ± 1.83	321.70 ± 78.84*	70.36 ± 21.89**

* $p = 0.014$, ** $p = 0.028$

Table 3. Serous TNF-α of swines in both groups (pg/mL)

Items	pre-herniorrhaphy	1 week post-herniorrhaphy	4 weeks post-herniorrhaphy
Group of allograft patch	5.69 ± 2.72	54.33 ± 8.17*	10.76 ± 8.87**
Group of polypropylene patch	4.32 ± 2.76	94.67 ± 13.02*	12.99 ± 10.85**

* $p = 0.008$, ** $p = 0.039$

crosslinking (*22*); however, crosslinking using glutaraldehyde yields unstable acetalization and produces toxic glutaraldehyde around the tissues, making it very difficult for host tissue to fuse and grow within the implanted animal tissue. Furthermore, acetalization does not completely remove foreign antigens. This is one of the bottlenecks that prevented the rapid development and advancement of biological materials across the world.

A new technique (Patent Number: US 6, 101, 555 and US 6, 231, 614B1) was developed to eliminate the toxic glutaraldehyde and completely remove the foreign antigens. Using a highly reactive epoxide as a fixation and crosslinking reagent, this epoxide can form stable cross bonds with protein, which significantly improves stability and eliminates toxicity. Moreover, the technique can induce the growth of host tissues into the implanted tissues, leading to the fusion of self tissue with implanted materials, which was confirmed by current results, 6 months after tension-free herniorrhaphy, the former regularly-aligned collagen degenerated and was broken, and many fibroblasts and fibrocytes were observed in the implanted allograft patch. 12 months after tension-free herniorrhaphy, new collagen fibers appeared and presented regularly, and there were no relapsed cases.

In addition, the technique involving modification of the protein molecules can also improve the physical mechanical parameters of the allograft patch. The tensile strength of the allograft patch was 90-160 N/cm, and the stretch rate was 62-82% according to the results. The maximum tensile strength placed on the abdominal wall in healthy adults ranges from 11 N/cm to 27 N/cm (*23*), and the bursting forces of the standard polypropylene patch have been measured at 40 to 100 N/cm (*24*). At 16 N/cm, the abdominal wall distends about 25%; however, polypropylene patches displayed a value of only 4-16% strain (*25*), which was possibly responsible for postoperative complaints of discomfort. The tensile strength of the biological allograft patch was almost 4 times maximum tensile strength of the abdominal wall in healthy adults, and was larger than that of the polypropylene patch, the stretch rate was also larger than that of the polypropylene patch and the abdominal wall.

Our current results revealed that all 9 swines implanted with allograft patches survived and were cured after tension-free herniorrhaphy without local infection. Serous IL-1β and TNF-α were both increased after herniorrhaphy, results of the IL-1β and TNF-α group of polypropylene patches were significantly higher than that of the group of allograft patches. TNF-α is a cytokine involved in inflammation, and IL-1β is a member of the interleukin 1 cytokine family, which is an important mediator of the inflammatory response. Thus allograft patches produced significantly lower inflammation to the host when compared to polypropylene patches.

$CD4^+$ T cells are also known as T helper cells, and $CD8^+$ T cells are also known as cytotoxic T cells, both $CD4^+$ and $CD8^+$ T cells are important in cellular immune responses and are also implicated in transplant rejections (*26*). The proportions of serous $CD4^+$ T cells and ratios of $CD4^+$/ $CD8^+$ T cells increased 1 week post-herniorrhaphy with allograft patches, which was not significant. The results of 4 weeks after herniorrhaphy were not significantly different when compared to that of pre-herniorrhaphy and 1 week post-herniorrhaphy. The allograft patches revealed satisfactory biocompatibility according to the results.

Ideal patches for tension-free herniorrhaphy are biological patches with similar components to

host tissues. They should also meet the following requirements (*27-29*): (1) stable compatibility without inflammation; (2) sufficient physical strength; (3) scaffold provision for self regeneration; (4) non-toxic and non-carcinogenic; (5) easy to produce, sterilize, preserve and tailor. Our results strongly indicate that the allograft patches used in the present study are promising for overcoming complications induced by macromolecular or composix patches, *e.g*. chronic, long-term pain, foreign body sensations, conglutination and so on and are a candidate for an ideal patch for tension-free herniorrhaphy.

5. Conclusion

The tissue engineering modified allograft patch with satisfactory biocompatibility is eligible and sufficient in tension-free herniorrhaphy of swine, and is a candidate for ideal patches for tension-free herniorrhaphy. Clinical trials should be performed for further evaluation of the allograft patch.

Acknowledgements

The authors thank Huiyan Huang from animal experiment center of Grandhope Biotech Co., Ltd., Guangzhou, China, for her assistance in preoperative preparation of swines. This project was supported by National Natural Science Fund for Young Scholars of China (81000177, Yuesi Zhong), Guangzhou province Technology Support Fund (Serial no.: 2006B60501007) and supported in part by Japan China Sasakawa Medical Fellowship.

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(*Received October 17, 2012 ; Revised November 27, 2012; Accepted December 2, 2012*)

Letter

DOI: 10.5582/bst.2012.v6.6.340

Proposed interaction between angiotensinogen and retinoblastoma tumor suppressor protein: Potential molecular origin of hypertension

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Keywords: Hypertension, angiotensinogen, retinoblastoma protein, LXCXE-like motif, peptides

Summary: *Hypertension ranks among the most important disease challenges on a global scale. Here, a novel hypothesis is presented which implicates angiotensinogen, i.e. the precursor protein for the hypertensive peptide angiotensin II, as a key culprit in the pathogenesis of hypertension. This hypothesis more precisely entails that intracellular angiotensinogen binds and thereby inactivates the retinoblastoma tumor suppressor protein (RB), consequently leading to an inflammatory and hyperproliferative state that significantly contributes to pathologically increasing blood pressure. Accordingly, a conceivable antihypertensive strategy could comprise RB-derived compounds that neutralize angiotensinogen.*

Cardiovascular disorders are still the most common cause for morbidity and mortality in industrialized countries. Within this major group of diseases, hypertension plays a leading role. Similar to other important pathologies such as cancer, the prevalence of hypertension increases with aging (*1*). Currently, among the principal treatments for hypertension are calcium-channel blockers, β-adrenergic antagonists and substances interfering with the renin-angiotensin system such as angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers (*1*). However, it is also known that the therapy of hypertension needs further improvement since it is not infrequent that polypharmacy by means of several different antihypertensive agents is needed to control certain forms of hypertension and, moreover, other (malignant) variants of hypertension are refractory to antihypertensive drug treatment.

As a result, the present investigation has aimed for identifying novel antihypertensive drug targets. In this context, I have focused on the potential pathogenetic role of angiotensinogen, *i.e.* the precursor protein for the hypertensive peptide angiotensin II, that has previously been described to occur in the intracellular compartment of human astrocytes (*2*) and of human dopaminergic neurons of the substantia nigra compacta (*3*).

Discovery and hypothesis: In the course of analyzing the amino acid sequence of human angiotensinogen, I have now discovered that it harbors the LXFXE amino acid motif (Figure 1) that has previously been defined as a retinoblastoma tumor suppressor protein (RB) binding motif in the viral oncoprotein Tax (*4*).

Leu Leu **Phe** Glu **Glu**
HTLV-I Tax oncoprotein residues 306-310

Leu Asp **Phe** Thr **Glu**
Human angiotensinogen residues 239-243

Figure 1. Alignment of LXFXE amino acid motifs in HTLV-1 Tax oncoprotein and human angiotensinogen. The crucial residues of the LXFXE motif have been highlighted in bold letters.

This novel finding suggests that intracellular angiotensinogen physically interacts with RB and thereby inactivates it, thus leading to cellular hyperproliferation and tissue inflammation both of which ultimately contribute to pathologically increasing blood pressure.

This new hypothesis on a potential involvement of angiotensinogen in the pathogenesis of hypertension is consistent with several reports on the inflammation-hypertension connection (*5-7*) along with data showing that the pro-inflammatory cytokine interleukin 6 increases the (plasma) level of angiotensinogen (*8*) and, moreover, that the angiotensinogen gene is a target for the intracellular pro-inflammatory protein NF-κB (*9*). In this context, it is worth noting that NF-κB inactivates RB (*10*) and, conversely, intact RB inhibits the gene regulatory activity of NF-κB through the conformational induction of a transcriptionally inactive NF-κB-DNA complex (*11*).

Given this now proposed candidate role for angiotensinogen in the etiology of hypertension, it should be promising to target the above-described angiotensinogen LXFXE RB-binding motif by means of antiproliferative

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RB-derived peptides such as those (anticancer) RB peptides that have previously been reported to recognize and neutralize the related LXCXE RB-binding motif (*12-16*).

This potential therapy of hypertension is in line with previous concepts on the inhibition of vascular smooth muscle cell proliferation, which is a crucial process in the pathology of hypertension, by means of an antiproliferative RB isoform (*17*) and the α_1-adrenergic receptor antagonist doxazosin (*18*).

Moreover and interestingly, the presently proposed treatment of hypertension by previously validated anti-cancer compounds such as MCR peptides (targeting the LXCXE motif) resembles the activity spectrum of naturally occurring substances such as quercetin that is *e.g.* a component of apples. Accordingly, quercetin has been shown to both block cancer cell growth (*19*) and to reduce blood pressure in hypertensive subjects (*20*).

If effective, the presently advanced therapeutic approach would once again underscore the previously recognized importance of *intracellular* targets and pathways for the successful treatment of human diseases (*21-24*). Last not least, this proposed (quercetin-like) strategy has the potential to additionally validate the likely effectiveness of both bionic and epigenetic interventions towards solving long-standing and important medical challenges.

Acknowledgments

The aim of the present hypothesis is both to stimulate research into a fundamentally new direction in the field of hypertension and to commemorate the 20th anniversary of the foundation of *Molecular Concepts Research* (*MCR*).

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(*Received November 19, 2012; Revised November 30, 2012; Accepted December 6, 2012*)

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Song PP, Gao JJ, Kokudo N, Dong JH, Tang W
2012; 6(3):147-152. (DOI: 10.5582/bst.2012.v6.3.147)

Letter

Proposed interaction between angiotensinogen and retinoblastoma tumor suppressor protein: Potential molecular origin of hypertension.
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(*Revised October 2011*)

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