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Brief Report

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Osteogenic differentiation of human ligament fibroblasts induced by conditioned medium of osteoclast-like cells

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Summary **Osteoclasts secrete factors that may promote mesenchymal stem cell mineralization *in vitro*. Fibroblasts are the most common cells in connective tissue and are involved in the process of exotic ossification in many diseases such as ankylosing spondylitis. The purpose of this study was to investigate whether osteoclast-like cells would induce the osteogenic differentiation of fibroblasts *in vitro*. In the present study, osteoclast-like cells (OLCs) were generated by $CD14^+$ cells from human peripheral blood. Fibroblasts were primarily cultured from spinal ligaments. After treatment with conditioned medium of OLCs, the level of alkaline phosphatase (ALP) and mineralization of fibroblasts increased significantly. cDNA microarray analysis identified a set of differentially expressed mRNA associated with signal transduction, cell differentiation, and bone formation, and microarray analysis of microRNA expression profiles revealed a group of microRNAs, including hsa-miR-20a, hsa-miR-300, hsa-miR-185, hsa-miR-30d, hsa-miR-320a, hsa-miR-130b, hsa-miR-33a, hsa-miR-155, and hsa-miR-222, that were significantly down-regulated. These microRNAs were predicted to have an inhibitory effect on genes associated with osteogenic differentiation, such as BMP2, Osteocalcin, and Runx2. The current results suggest that osteoclasts might induce the osteogenic differentiation of fibroblasts *in vitro* and that miRNA may play an important role in regulation of the cell-cell interaction between osteoclasts and fibroblasts.**

Keywords: Mineralization, cell-cell interaction, mRNA, microRNA, microarray

1. Introduction

The osteoclast (OC) is a tissue-specific macrophage polykaryon derived from the monocyte/macrophage precursor cells at or near the bone surface (*1*). Fibroblasts are the most common cells of the connective tissue and are derived from primitive mesenchyme (*2*,*3*). Fibroblasts are mainly responsible for the production and turnover of extracellular matrix (ECM), which is rich in collagen and other macromolecules, and play an important role in trauma repair and pathologic ectopic ossification in some diseases, such as ankylosing spondylitis and systemic sclerosis (*3*,*4*). The mechanisms of osteogenic differentiation of fibroblasts and ossification have not been clarified. Fibroblasts and osteoblasts are from the same lineage (*5*). The widely accepted view is that osteoclasts can promote the differentiation of osteoblasts. Conditioned medium derived from human osteoclasts induces preosteoblasts to form bone-like nodules (*6*). Gene array analysis, in combination with loss-of-function studies, revealed that mature osteoclasts secrete Wnt10b, sphingosine 1-phosphate, and bone morphogenetic protein-6 (BMP6) and might promote osteoblast mineralization (*7*). Thus, osteoclasts may play a key role in the process of inducing heterotopic ossification mediated by fibroblasts. The objective of the present study was to evaluate the effect that osteoclasts have on inducing the osteogenic differentiation of fibroblasts and initially investigate the mechanism by which this occurs.

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2. Materials and Methods

2.1. *Primary culture of ligament fibroblasts*

Interspinous ligaments were obtained with the informed consent of patients with lumbar spondylolisthesis. Fibroblasts were primarily cultured from ligaments with the collagen collagenase digestion method using collagenase I (Sigma-Aldrich China, Shanghai, China). Fibroblasts were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (HiClone, Logan, Utah, USA) containing 10% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, California, USA), 100 U/mL penicillin (Invitrogen, Carlsbad, California, USA), and 100 μg/mL streptomycin (Invitrogen, Carlsbad, California, USA) and were incubated in a humidified atmosphere (37°C, 5% CO_2). The culture medium was changed after 24 h and every other day thereafter.

2.2. *Generating osteoclast-like cells from peripheral blood*

Peripheral blood was drawn from normal volunteers and supplemented with the anticoagulant ethylenediam inetetraacetic acid (EDTA). $CD14^+$ cells were isolated by Ficoll-Paque (GE Healthcare, Salt Lake City, Utah, USA) density gradient centrifugation followed by isolation with anti-CD14 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). This was done in accordance with the manufacturer's instructions. The isolated $CD14^+$ cells were seeded in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Five × 10^5/well $CD14^+$ cells were added in 24-well containing either bovine bone slices (8 mm × 8 mm × 0.2 mm, sterilized by ultraviolet radiation) or glass cover slips (8 mm × 8 mm) and incubated at 37°C with 5% CO_2 in a humidified incubator. The medium was supplemented with 25 ng/mL macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, New Jersey, USA) and 30 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) (PeproTech, Rocky Hill, New Jersey, USA). Cultures were incubated and culture medium containing the factors was replenished every 3 d. After 21 d of culturing, the characteristics of osteoclasts were identified. Using the same method, more osteoclasts were cultured in a 25 cm^2 cell culture flasks (without bovine bone slice or glass cover slips) in order to harvest conditioned medium.

Observation of morphological characteristics: An inverted phase contrast microscope was used to observe the characteristics of multinucleate cells. Tartrate-resistant acid phosphatase (TRAP) staining: Histochemical staining for TRAP, a marker of osteoclasts, was carried out with an Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich China, Shanghai, China). Assessment of bone resorption function: Bovine bone slices with cultured OLCs were subjected to fixation, dehydration, isoamyl acetate replacement, routine critical point drying, and plating platinum. Cells were then observed and photographs were taken with a Hitachi S570 scanning electron microscope.

2.3. *Conditioned medium of osteoclast-like cells treatment*

After OLCs were cultured for 21 d, addition of M-CSF and RANKL to medium was halted and the medium was refreshed every 2 d. The conditioned medium was collected and centrifuged to remove cells that were suspended in the medium. Fibroblasts were conditioned in OLC-conditioned medium and medium was refreshed every 2 d. Fibroblasts were cultured in normal medium (DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, without M-CSF and RNAKL) as a control.

2.4. *Assessment of fibroblast mineralization*

After fixation with 95% alcohol, a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Nantong Jiangsu, China) was used to detect the expression of ALP. Cultures were fixed and stained for mineralization with Alizarin red. Alizarin red was eluted with 10% cetylpyridinium chloride, and levels were quantified in comparison to a standard curve. To obtain a normal distribution and equal variance between the two test groups (four wells of OLC-conditioned medium (OCMT) fibroblasts and four wells of control fibroblasts), all data were $\log_{10}$-transformed. When only two groups were compared, an unpaired Student's *t*-test was used to test significance.

2.5. *Microarray detection of changes in the mRNA and miRNA expression profiles of fibroblasts*

2.5.1. *mRNA microarray*

Total RNA from each sample was quantified using the NanoDrop 1000 and RNA integrity was assessed using standard denaturing agarose gel electrophoresis. About 5 μg of total RNA from each sample was used for labeling and array hybridization in the following steps: 1) Reverse transcription with an Invitrogen Superscript ds-cDNA synthesis kit; 2) ds-cDNA labeling with a NimbleGen one-color DNA labeling kit; 3) Array hybridization using the NimbleGen Hybridization System and then washing with the NimbleGen wash buffer kit; 4) Array scanning using the Axon GenePix 4000B microarray scanner (Molecular Devices Corporation). Scanned images (TIFF format) were then imported into NimbleScan software (version 2.5) for grid alignment and expression data analysis.

2.5.2. *miRNA microarray*

Total RNA are harvested using TRIzol (Invitrogen, Carlsbad, California, USA) and an RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. After RNA measurement using the NanoDrop instrument, the samples were labeled using the miRCURY™Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array (v.14.0). The samples were hybridized on a hybridization station following the scheme outlined in sample submission. Scanning was performed with the Axon GenePix 4000B microarray scanner. GenePix pro V6.0 was used to read the raw intensity of the image.

2.5.3. *Data analysis*

Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. The Probe level files and Gene level files were generated after normalization. The 2 gene level files were imported into Agilent GeneSpring GX software (version 11.5) for further analysis. Genes with values greater than or equal to the lower cut-off (50.0) in all samples ("All Target Values") were chosen for data analysis. Differentially expressed genes were identified through Fold Change filtering. Pathway analysis and GO Analysis were performed to reveal the biological functions of this subset of differentially expressed genes. Finally, hierarchical clustering was performed to provide distinguishable gene expression profiling among samples. microRNA targets were predicted by three programs: miRanda, PicTar, and Target-Scan. The overlap of the results predicted by three programs served as the acceptable target genes of microRNA.

3. Results and Discussion

3.1. *Primary cultured fibroblasts and OLCs with characteristics of osteoclasts*

Fibroblasts were generated by spinal ligaments (Figure 1A). After 21 d of induction of M-CSF and RANKL, osteoclast-like cells had the characteristics of osteoclasts, such as a large volume, irregular shape, pseudopodium and multinucleation (Figure 1B), and high TRAP positivity (Figure 1C). Under SEM observation, osteoclast-like cells were found to have bone resorption ability since there were absorption lacunae below the osteoclasts (Figure 1D).

3.2. *Osteogenic differentiation of fibroblasts induced by OLC-conditioned medium*

After 21 d of induction with OLC-conditioned medium, the ALP positivity of fibroblasts was approximately 40% (Figure 2B). In contrast, the ALP

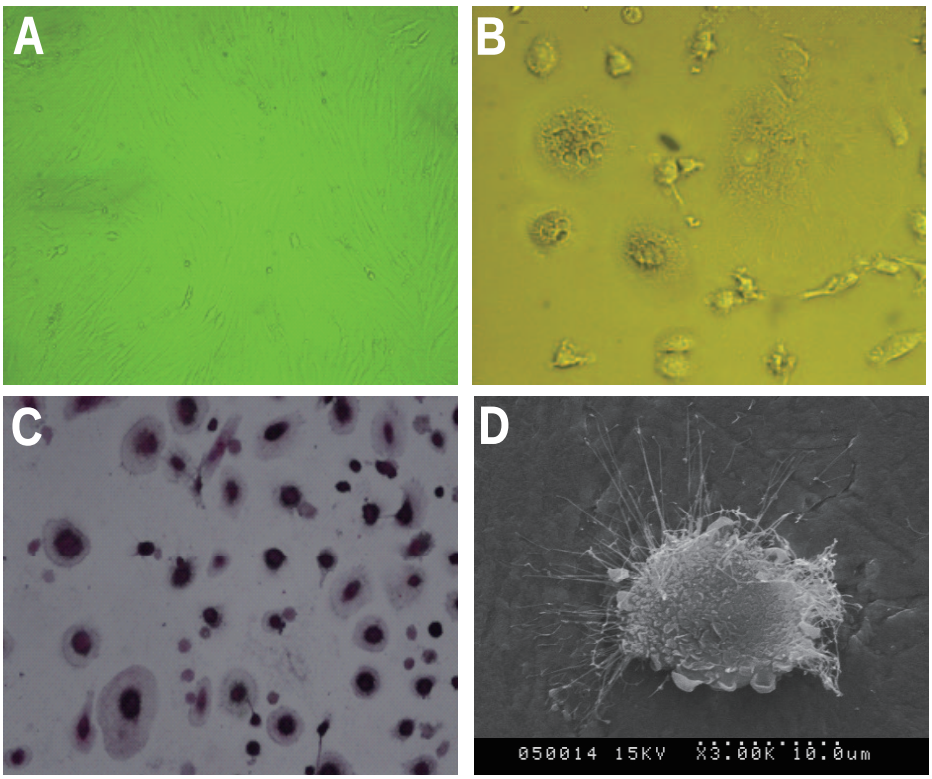

Figure 1. Primary cultured ligament fibroblasts and osteoclast-like cells generated by peripheral blood mononuclear cells. **(A)** Fibroblasts generated by ligaments; **(B)** Multinuclear osteoclast-like cells; **(C)** TRAP staining of OLC; **(D)** Bone resorption of OLC.

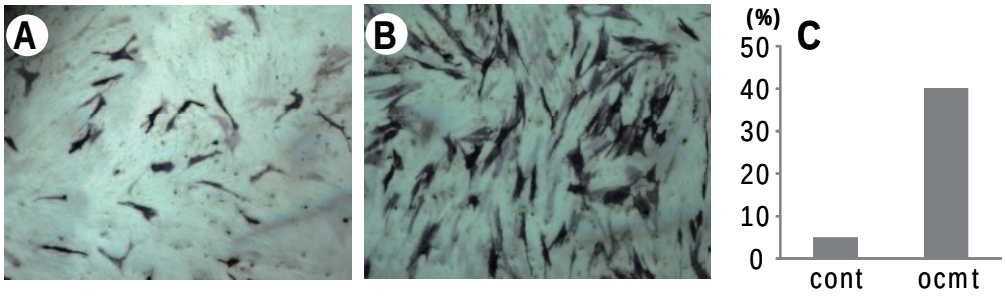

Figure 2. Results of Alkaline Phosphatase Color Development. (A) Control; **(B)** Fibroblasts treated with OLC-conditioned medium; **(C)** Comparison of ALP positivity of the control and fibroblasts treated with OLC-conditioned medium. $p < 0.05$.

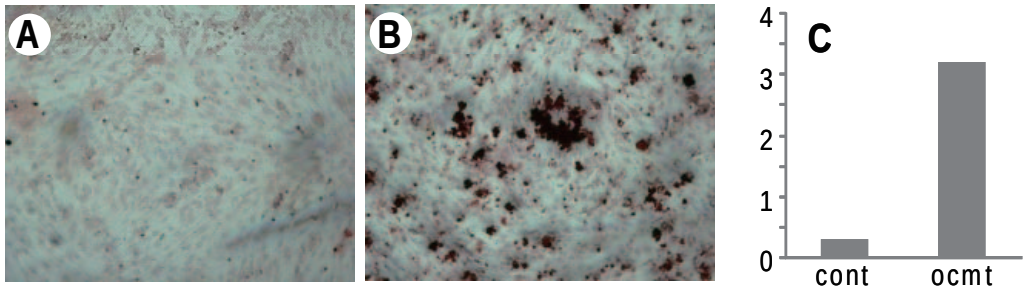

Figure 3. Effect of osteoclast-conditioned media on fibroblast mineralization. (A) Control; **(B)** Fibroblasts treated with OLC-conditioned medium; **(C)** Comparison of the mineralization of control and fibroblasts treated with OLC-conditioned medium. $p < 0.05$.

positivity of the control group was only 5% (Figure 2A). Conditioned media from osteoclast-like cells significantly increased mineralization of fibroblast cultures (Figure 3).

3.3. *mRNA microarray data analysis*

Compared to fibroblasts cultured in normal medium, fibroblasts treated with OCMT had 522 genes up-regulated more than 2-fold and 415 genes down-regulated more than 2-fold. The scatterplot is a visualization of the variation between chips (Figure 4). GO analysis revealed that the differentially expressed genes were mainly involved in bioprocesses like response to stimulus, signal transduction, cell activation, myeloid cell differentiation, calcium-mediated signaling, cell adhesion, and immune response. The cellular components of differentially expressed genes were mainly distributed in MHC protein complex, plasmolemma, the extracellular region, extracellular space, collagen, and the like. The molecular function of differentially expressed genes was mainly related to MHC class II receptor activity, protein complex binding, signal transducer activity, chemokine activity, and collagen binding. Pathway analysis revealed that differentially expressed genes mainly participated in pathways, such as cell adhesion molecules, leukocyte transendothelial migration chemokine signaling pathways, cytokine-cytokine receptor interaction, and Toll-like receptor signaling pathways.

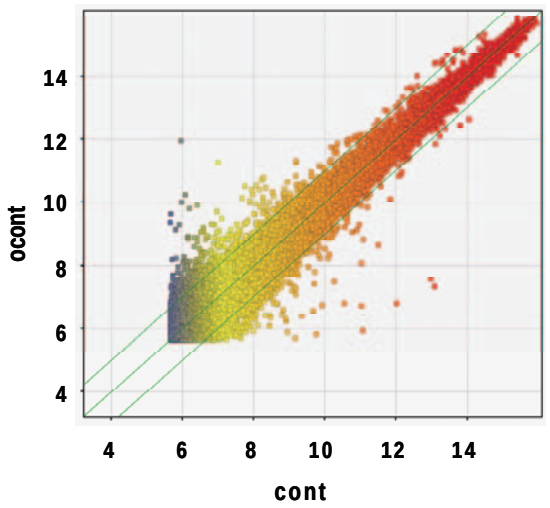

Figure 4. Scatter-plot for ocmt-cont (mRNA). Scatter-plot to assess the variation (or reproducibility) between mRNA chips. The axes of the scatter-plot are the averaged normalized signal values of the samples in each group (log_2 scaled). The green lines are Fold Change Lines (The default fold change value given is 2.0). Genes above the top green line and below the bottom green are 2-fold change genes.

3.4. *miRNA microarray data analysis*

Compared to fibroblasts cultured in normal medium, fibroblasts treated with OCMT had 28 miRNAs up-regulated more than 2-fold and 59 miRNAs down-regulated more than 2-fold. Target genes prediction software analysis revealed that among the down-regulated miRNAs the target genes of miR-20a and hsa-miR-300 included BMP2. Osteocalcin (bone

gamma-carboxyglutamate protein, BGLAP) was one of the target genes of hsa-miR-185 while hsa-miR-30d, hsa-miR-320a, hsa-miR-20a, hsa-miR-130b, hsa-miR-33a, hsa-miR-155, and hsa-miR-222 all targeted Runx2. These genes are all related to osteogenic differentiation.

Mature osteoclasts secreted products that promote fibroblast mineralization and caused a series of changes in mRNA and miRNA expression profiles of fibroblasts. Through cytokine-cytokine receptor interaction and regulation of signaling pathways, the products secreted by osteoclast-like cells may affect the metabolism and differentiation of fibroblasts. The current microarray analysis suggests that the Toll-like receptor signaling pathway may play an important role in these processes. A recent study has found that the Toll-like receptor is associated with osteogenic differentiation of stromal cells (*8*).

No previous reports have described the effects of osteoclasts on fibroblasts, but studies have found that fibroblasts can induce differentiation of precursor cells into mature osteoclasts. Bloemen and colleagues (*9*) found that gene expression of intercellular adhesion molecule-1 (ICAM-1) and osteoclastogenesis-related genes (RANKL, RANK, TNF-a, and IL-1b) was highly up-regulated in co-cultures with fibroblasts. The current study has shown that conditioned medium of osteoclast-like cells can influence the adhesion molecule pathway of fibroblasts. Hypoxia-induced ID-2 may contribute to joint destruction in RA patients by promoting synovial fibroblast-dependent osteoclastogenesis (*10*). Moreover, oestrogen can inhibit osteoclast formation induced by periodontal ligament fibroblasts (*11*). The current study suggests that there is interaction between osteoclasts and fibroblasts. Fibroblasts can induce osteoclastogenesis and osteoclasts induce osteogenic differentiation of fibroblasts. These findings will help to understand the pathomechanism of ectopic ossification mediated by fibroblasts.

miRNAs are small noncoding RNAs that significantly regulate the translation of protein coding genes in higher organisms (*12-14*). These small RNAs (approximately 22 nucleotides) are involved in almost every biological process, including early development, lineage commitment, growth and differentiation, cell death, and metabolic control (*12-16*). Each miRNA targets an average of 100-200 genes by binding preferentially to their 3' UTRs by means of partial sequence complementarity (*17*). miRNAs engage in a wide diversity of biological processes by coordinating with transcription factors, and this kind of cross-layer coregulation may have greater specificity than intra-layer coregulation (*18*). The current study has suggested that microRNA plays an important role in the osteogenesis of mesenchymal stem cells (*19*). Changes in microRNA expression profiles were examined in the current study during the process of osteogenic differentiation induced by OLC-conditioned medium. This study also found that microRNAs such as hsa-miR-20a, hsa-miR-300, hsa-miR-185, hsa-miR-30d, hsa-miR-320a, hsa-miR-130b, hsa-miR-33a, hsa-miR-155 and hsa-miR-222 may play a key role by regulating the expression of BMP2, Osteocalcin, Runx2, in the process of osteogenic differentiation.

The present study investigated the effects of osteoclast-like cells on induction of fibroblast mineralization without direct cell-to-cell contact. This study also identified a set of mRNA and miRNA differentially regulated during fibroblast mineralization. However, the factors secreted by osteoclast-like cells that influence signal transduction and that regulate expression miRNA and their target genes remain unclear. More research is needed to determine the precise mechanism of these processes.

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Brief Report

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Effect of c-Met inhibitor SU11274 on hepatocellular carcinoma cell growth

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Summary **c-Met, a type of receptor tyrosine kinase, may be significantly associated with the progression of hepatocellular carcinoma (HCC). In addition, des-γ-carboxyprothrombin (DCP) has been found to interact with c-Met and activate HCC cell growth. Therefore, the functional inhibition of c-Met expressed on HCC cells should arrest HCC cell growth. The present study found that the c-Met inhibitor SU11274 suppressed HCC cell growth by inhibiting the activation of c-Met. Furthermore, this inhibitor also neutralized the activation of HCC cell growth resulting from the addition of DCP. These results suggest that the functional inhibition of c-Met might be a target for the development of chemotherapeutic agents for HCC, and especially those that are positive for expression of DCP.**

Keywords: c-Met, hepatocellular carcinoma (HCC), inhibitor, growth, des-γ-carboxyprothrombin (DCP)

1. Introduction

c-Met is a type of receptor tyrosine kinase expressed on the membranes of various cells (*1*). Hepatocyte growth factor (HGF), also known as scatter factor (SF), has been identified as the ligand for c-Met (*2*), and their interaction can activate the kinase activity of c-Met to trigger various cellular events, *e.g.* tissue regeneration, cell proliferation, and invasion (*3*). The phosphorylation of c-Met and subsequent activation of various downstream signal transduction pathways may play a significant role in the progression of hepatocellular carcinoma (HCC) (*4-6*). Several clinical studies noted overexpression of c-Met in HCC tissue compared to surrounding non-cancerous liver tissue (*7-9*), and this overexpression was suggested to correlate with worse behavior of HCC (*8*). Furthermore, the down-regulation of c-Met expression by transfecting siRNA suppressed the proliferation and invasion of an HCC cell line (*10,11*). Thus, the expression of c-Met was found to be associated with the progression of HCC and its inhibition may contribute to the development of chemotherapy for HCC. Little research, however, has analyzed the inhibitory effect of c-Met on HCC cells by way of small-molecular compounds.

Des-γ-carboxyprothrombin (DCP), also referred to as protein induced by vitamin K absence or antagonist II (PIVKA-II), is known to be an effective tumor marker for HCC (*12-14*). This tumor marker is regularly used in clinical diagnosis to screen patients with primary HCC and to monitor the recurrence of HCC after curative therapy (*15,16*). While extensive scientific evidence has been assembled to indicate the clinicopathological significance of DCP, a high level of DCP in the sera of patients with HCC was significantly associated with the presence of vascular invasion followed by the presence of intrahepatic metastasis (*16-18*). Thus, DCP may serve to predict a worse prognosis of patients with HCC. Additionally, recent molecular biological studies on DCP have

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revealed that DCP activates HCC cell growth (*19*,*20*). Gao *et al*. demonstrated that the proliferation of HCC cells increased as a result of DCP in a dose- and time-dependent manner (*19*). In addition, this action of DCP was also detected *in vivo* analyses using a nude mouse model with HCC cell xenograft (*19*). A biochemical study performed by Suzuki *et al.* showed that DCP can increase the DNA synthesis of HCC cells *via* the activation of c-Met-Janus kinase (JAK)-signal transducers and activators of the transcription 3 (STAT3) pathway (*20*). Although previous studies have suggested that the expression and activation of c-Met may play important roles in the growth of HCC cells, and especially those induced by DCP, c-Met inhibitor has never been analyzed in terms of the suppression of HCC cell growth activated by DCP.

The aim of the present study was to clarify the inhibitory effect that a small-molecular inhibitor of c-Met had on HCC cell growth and its relationship to DCP.

2. Materials and Methods

2.1. *Compound*

A small-molecular c-Met inhibitor named SU11274 ((3Z)-*N*-(3-chlorophenyl)-3-({3,5-dimethyl-4-[(4-methylpiperazin-1-yl)carbonyl]-1H-pyrrol-2-yl}methylene)-*N*-methyl-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide, Mol. Wt. = 568.09) was purchased from Calbiochem, Inc. (San Diego, CA, USA) (*21*). This compound was dissolved in dimethyl sulfoxide (DMSO) solution so that the concentration of DMSO was less than 0.1% (v/v) in the analyzed samples.

DCP was generously donated by Eisai Co., Ltd., Tokyo, Japan. DCP was purified from the conditioned media of the DCP-producing cell line PLC/PRF/5 by affinity chromatography with an anti-prothrombin antibody. DCP was distinguished from normal prothrombin using high-performance liquid chromatography (HPLC) (*20*).

2.2. *Cell lines*

$HepG_2$ (a well-differentiated HCC cell line) cells were obtained from European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). HuH-7 (a well-differentiated HCC cell line) and HLE (poorly-differentiated HCC cell line) cells were obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan). These cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a humid atmosphere (5% CO_2-95% air) and were harvested by brief incubation in Enzyme-free Cell Dissociation Solution (Millipore Co., Bedford, MA, USA).

2.3. *Cell growth assay*

Continuously cultured $HepG_2$, HuH-7 and HLE cells were harvested in tubes and resuspended in DMEM containing 10% FBS after they were washed with PBS. The cells were seeded in triplicate in 96-well plates at a density of 6×10^3 cells in 100 μL with or without a concentration of SU11274 and/or DCP and incubated for 24 and 72 h at 37°C in a 5% CO_2 atmosphere. Cell viability was evaluated using a methylthiazole tetrazolium (MTT) cell proliferation assay kit in accordance with the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). The IC_{50} (concentration of drug that inhibits cell growth by 50%) value was calculated using SPSS 11.5 software.

2.4. *Western blot analysis*

Continuously cultured $HepG_2$, HuH-7 and HLE cells were harvested in tubes and resuspended in DMEM containing 10% FBS after they were washed with PBS. The cells were seeded in triplicate in 6-well plates at a density of 3×10^5 cells in 2 mL with or without a concentration of SU11274 and incubated for 48 h at 37°C in a 5% CO_2 atmosphere. Then, the cells were harvested in tubes and total cell lysates were prepared as previously described (*22*). Protein concentrations of supernatant were determined using the DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Thirty μg of total cellular proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) transfer membranes by Western blotting. Rabbit anti-human c-Met polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human phosphorylated-c-Met polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-human phosphorylated-extracellular signal-regulated kinase (ERK) polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA), and mouse anti-human β-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) were used as primary antibodies.

2.5. *Statistical analysis*

Statistical analysis was performed with the Student's *t*-test using SPSS 11.5 software. $p < 0.05$ was indicative of a significant difference. All experiments were performed in triplicate.

3. Results and Discussion

First, the inhibitory effect of c-Met inhibitor SU11274 on HCC cell growth was analyzed. As shown in Figure 1, the growth of all HCC cell lines analyzed was inhibited by the addition of SU11274. This inhibitory

effect was particularly apparent with over 2.5 μM of SU11274. This inhibitory effect occurred in a time- and dose-dependent manner but differed among HCC cell lines with 48 and 72 h of incubation. The growth of HLE cells was gradually inhibited by a lower concentration of SU11274 (0.0625-1.25 μM) although those concentrations of SU11274 had no significant effect on the growth of $HepG_2$ and HuH-7. However, similar IC_{50} values were noted for the cell lines analyzed; 8.16 μM SU11274 and 48 h of incubation and 6.75 μM SU11274 and 72 h of incubation inhibited $HepG_2$ cells, 8.24 μM SU11274 and 48 h of incubation and 8.08 μM SU11274 and 72 h of incubation inhibited HuH-7 cells, and 6.36 μM SU11274 and 48 h of incubation and 7.20 μM SU11274 and 72 h of incubation inhibited HLE cells. These results suggest that the addition of the small-molecular c-Met inhibitor SU11274 may inhibit the growth of HCC cells.

Next, Western blot analysis was performed to clarify the changes in c-Met expression or its activation profile as a result of SU11274. All HCC cell lines analyzed were positive for c-Met expression, and this level of expression in those HCC cell lines did not change significantly with the addition of SU11274 (Figure 2). In contrast, the expression of phosphorylated-c-Met gradually decreased in a dose-dependent manner with SU11274. The rate of inhibited expression of phosphorylated-c-Met in the presence of 5 μM SU11274 was 43%, 84%, and 53% for $HepG_2$, HuH-7, and HLE cells, respectively. In addition, the expression of phosphorylated-ERK, the downstream kinase of the signal transduction pathway activated by c-Met, also decreased in a dose-dependent manner with SU11274. The rate of inhibited expression of phosphorylated-ERK in the presence of 5 μM SU11274 was 18%, 39%, and 78% for $HepG_2$, HuH-7, and HLE cells, respectively. These results suggest that the down-regulation of c-Met activation by SU11274 may inhibit the growth of HCC cells.

As described previously, c-Met may act as a target receptor of DCP and trigger the activation of HCC cell growth (*20*). Therefore, the inhibition of c-Met presumably suppresses HCC cell growth induced by DCP. The present study investigated the ability of SU11274 to inhibit DCP by simultaneously

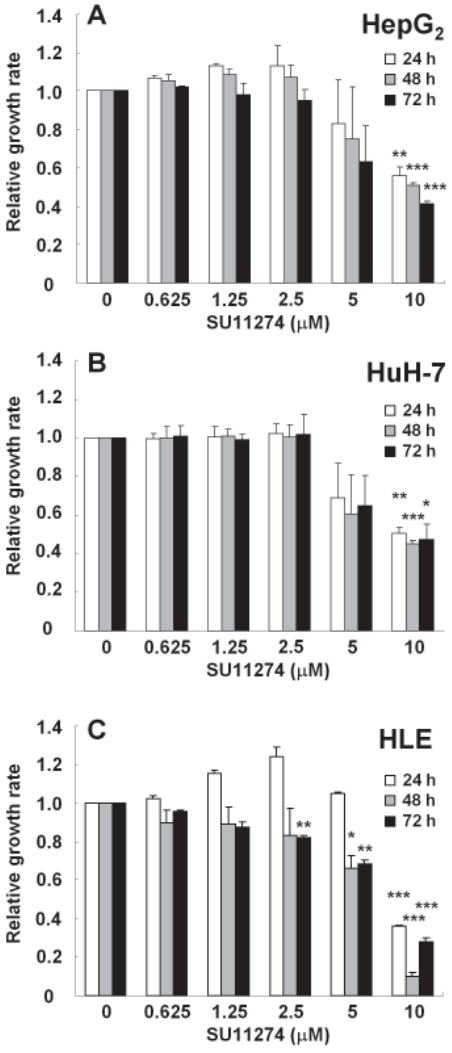

Figure 1. Relative growth rates of (A) $HepG_2$, (B) HuH-7 and (C) HLE cells in the various concentrations of SU11274. White column, 24 h; gray column, 48 h; solid column, 72 h incubation with SU11274. The data represent mean ± S.D. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs.* control with 0 μM SU11274.

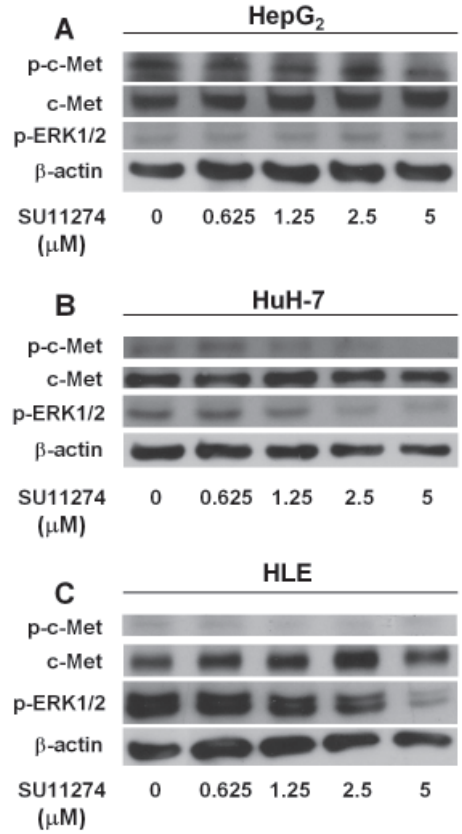

Figure 2. Expressed levels of phosphorylated-c-Met, overall c-Met, and phosphorylated-ERK in (A) $HepG_2$, (B) HuH-7 and (C) HLE cells detected by Western blot analysis.

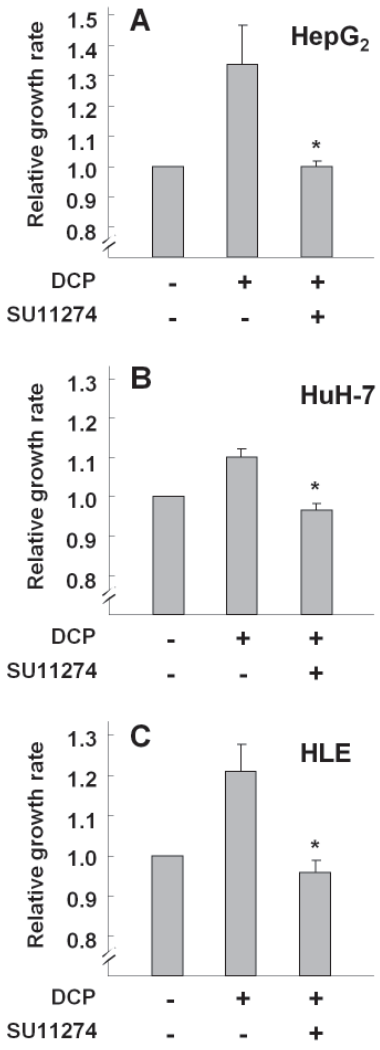

Figure 3. Relative growth rates of (A) $HepG_2$, (B) HuH-7 and (C) HLE cells in the presence of DCP (160 ng/mL) and/or SU11274 (2.5 μM). Whereas the growth of HCC cells was activated by the addition of DCP, the presence of SU11274 with DCP significantly inhibited this action. The data represent mean ± S.D. ($n = 3$). * $p < 0.05$ *vs.* sample with DCP.

incubating HCC cells with these substances for 48 h. The concentration of DCP was set at 160 ng/mL in accordance with the results of a previous study (*19*). In addition, the concentration of SU11274 was set at 2.5 μM because the inhibitory effect on cell growth was detected with 5 μM and 48 h of incubation (Figure 1). As a result, the incubation of HCC cells with DCP activated HCC cell growth, as shown in Figure 3. Previous studies also analyzed the effect of DCP on HCC cell growth using $HepG_2$ (*19*) and results of those studies coincided with the results of the present study. The results indicated that the relative extent of growth in the presence of DCP differed among the HCC cell lines analyzed, and this difference may be due to their sensitivity to DCP. The effect of SU11274 on DCP was also analyzed. The activation of HCC cell growth by DCP was neutralized by the simultaneous addition of SU11274 (Figure 3). These results indicate that the growth of HCC cells was up-regulated by DCP *via* the activation of c-Met and that these biological events can be inhibited by c-Met inhibitor.

This study was performed with the aim of evaluating the effectiveness of the c-Met inhibitor SU11274 as an anti-cancer chemotherapeutic agent in treating HCC cells. Results revealed that SU11274 had the ability to inhibit HCC cell growth. Several mechanisms whereby the activation of c-Met leads to HCC progression have been suggested. These mechanisms involve ERK-mediated and phosphatidylinositol 3 kinase (PI3K)-mediated pathways (*23*). As described previously, the JAK-STAT3 pathway may also play an important role in the up-regulation of HCC cell proliferation induced by DCP (*20*). Thus, various pathways triggered by activated c-Met might regulate the behavior of HCC. Further clarification of the c-Met-mediated mechanism of HCC progression may lead to the development of novel chemotherapeutic agents for HCC.

In conclusion, this study reported that inhibition of c-Met by the small-molecular inhibitor SU11274 arrested HCC cell growth. In addition, DCP's action as a growth factor might be neutralized by the inhibition of c-Met activation. Various researchers are developing novel c-Met inhibitors (*24*,*25*), and these inhibitors may prove of use in chemotherapy to treat HCC.

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Brief Report

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Basiliximab as therapy for acute rejection after liver transplantation for hepatitis C virus cirrhosis

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Summary **Steroid bolus therapy for acute rejection after liver transplantation for hepatitis C virus (HCV) cirrhosis often results in graft loss due to adverse effects. The efficacy and safety of basiliximab for the treatment of acute cellular rejection (ACR) in adult liver transplantation has not been adequately evaluated. Three patients received basiliximab as rescue therapy for acute rejection. The outcome and biochemical parameters were recorded before and after treatment with basiliximab. These results were compared to 11 patients who received steroid therapy for ACR. The median time from transplantation to the development of ACR was 19 days (range, 9-49 days). The degree of ACR was mild or moderate. Resolution of rejection was obtained in all patients and the median time from the onset to resolution of ACR was 16 days (range, 6-41 days). A steroid resistant reaction occurred in 2 of 11 patients and OKT3 was used, and the rejection eventually resolved in all patients. Five patients died within 2 to 22 months after transplantation and four of them died from graft failure. In the basiliximab group, there were no significant immediate adverse effects. One patient died from pneumonia 8 months after transplantation. In conclusion: Basiliximab can be safely used as rescue therapy for ACR without significant adverse effects in patients who underwent liver transplantation for HCV cirrhosis.**

Keywords: Hepatitis C, donor, living donor liver transplantation

1. Introduction

Liver transplantation is an effective treatment for end-stage liver disease due to hepatitis C virus (HCV). Despite major advances in immunosuppression, acute cellular rejection (ACR) remains a significant postoperative issue. This can be a major risk factor for the development of chronic allograft rejection and graft loss. Despite the use of calcineurin inhibitors, ACR occurs in 36% to 57% of transplant recipients (*1*). Typical management involves optimization of baseline immunosuppression and methylprednisolone boluses. In addition, 28% to 35% (*2*) of patients do not respond to high-dose steroid therapy and require further intervention, including antithymocyte globulin or OKT3. Repeated high-dose steroids and OKT3, however, reduce graft survival as a consequence of severe HCV recurrence (*3*).

Basiliximab is a chimeric (human/mouse) monoclonal antibody that selectively binds to the α subunit (CD25) of the interleukin-2 (IL-2) receptor (*4*), preventing normal T-cell proliferation and thereby the progression of ACR (*5*). IL-2 promotes the proliferation of activated T cells by engaging the IL-2 receptor, and this has a crucial role in the mediation of ACR. The efficacy of basiliximab has been evaluated and has been shown to rescue the graft from ACR (*6*). In the present study, we examined the efficacy and safety of basiliximab as a therapeutic agent for rescue of ACR in liver recipients for HCV-related cirrhosis, in comparison with conventional high-dose steroid therapy.

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2. Patients and Methods

We performed 213 adult liver transplantations from 2004 to September 2010. Of these, 89 were transplanted due to HCV cirrhosis (4 patients co-infected with HIV were excluded). Of the 89 patients, 16 (18%) were complicated with ACR within 60 days after transplantation at the University of Tokyo Hospital. The ACR rate in the patients other than HCV during the same period was 29% (n = 294) which was higher than that of HCV patients without significant difference. After transplantation, all of the patients received the same immunosuppressive treatment with tacrolimus and methylprednisolone, as described previously (*7*). Briefly, target tacrolimus trough levels were 15 to 20 ng/mL during the first 2 weeks and 10 to 15 ng/mL thereafter. Intravenous methylprednisolone was started during the operation (20 mg/kg/day), and gradually tapered thereafter (7 days after transplantation, 0.75 mg/kg; two weeks, 0.35 mg/kg; one month, 0.3 mg/kg; two months, 0.25 mg/kg; three months, 0.2 mg/kg; 4 months, 0.2 mg/kg). The subjects comprised 12 men and 4 women who underwent liver transplantation with subsequent development of ACR. ACR was defined as a biopsy-proven episode and graded according to the Banff schema (*8*). The indication for liver biopsy was a significant increase in total bilirubin, aspartate, and alanine aminotransferase levels.

Until the end of 2007, the patients were treated with bolus intravenous methylprednisolone, regardless of ACR severity, at a starting dose of 20 mg/kg/day, as previously described (*9*). The dose was reduced by half each day and the therapy was continued for 5 days. When acute rejection was refractory to high-dose methylprednisolone therapy, a second bolus was then given with mycofenolate mofitile (CellCept, Roche Pharmaceuticals, Basel, Switzerland; 3 g/day). If the rejection was not cured by two rounds of steroid therapy the episode was diagnosed as steroid-resistant rejection and OKT3 was administered (Figure 1). After 2008, the regimen was changed. Basiliximab (two 20-mg doses with a 2-day inter-dose interval; Simulect, Novartis Pharmaceuticals, Basel, Switzerland) was added to the immunosuppressive treatment with tacrolimus and methylprednisolone. No bolus intravenous methylprednisolone was administered. The primary outcome measures were treatment success with resolution of ACR, as indicated by normalization of alanine aminotransferase or resolution of liver biopsy changes, or treatment failure due to ongoing rejection. No infectious prophylaxis was done. Immediate treatment of adverse effects and infectious complications within 1 month of treatment were recorded.

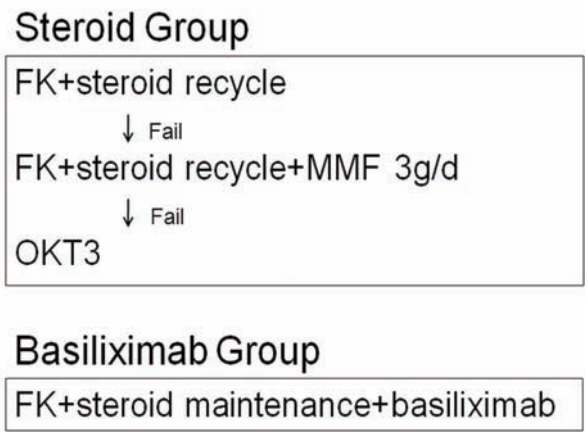

Figure 1. Regimens in the steroid and basiliximab groups. Abbreviations: FK, tacrolimus; MMF, mycophenolate mofetil.

3. Results and Discussion

The median time from transplantation to the development of ACR was 19 days (range, 9-49 days). The degree of ACR was mild or moderate. Median follow-up from transplantation was 25 months (range, 2-81 months).

Treatment outcomes are summarized in Table 1. Of the 11 patients in the steroid group, 2 had a steroid-resistant reaction and OKT3 was used. The overall rejection grade in the two patients was moderate. In a semi quantitative assessment, venous endothelial inflammation was scored as 2 (moderate). Rejection eventually resolved in all patients, which was confirmed by the subsequent biopsy in 7. Five patients died within 2 to 22 months after transplantation and four of them died from graft failure. In all three patients in the basiliximab group, rejection resolved without significant immediate adverse effects. In one patient the resolution was histologically confirmed (Banff rejection activity index changed from 4 to 2 after the therapy). One patient died of pneumonia 8 months after transplantation.

Aspartate aminotransferase levels normalized in all patients. The median time from the onset to resolution of ACR was 16 days (range, 6-41 days).

The present report confirms that basiliximab can be effective in reversing ACR in HCV patients. All patients with histologic evidence of ACR before treatment with basiliximab had resolution of ACR. Three patients with ACR were managed effectively with basiliximab without the addition of other immunosuppressive agents. Furthermore, none of these patients had recurrent episodes of ACR. In contrast, of the 11 patients complicated with ACR that received steroid pulse therapy, 4 died of graft failure, probably due to HCV recurrence, although ACR was successfully resolved in all cases.

The safety of basiliximab was previously reported, with no evidence of significant acute toxicity (*2,10*) or increased risk of infection (*11*). There have been

Table 1. Patients and treatment outcomes

Age, sex	ACR onset (d)	On the day ACR diagnosis		Number of steroid boluses	MMF	OKT	Interval for ACR resolution (d)	After the therapy		Outcome	Cause of death
		Banff score	Blood test (ALT/ALP/TB)					Banff score	Blood test (ALT/ALP/TB)		
Steroid group											
44, M	42	3	314/546/0.7	1	No	No	23	–	36/471/1.2	81A	–
57, M	11	4	86/521/1.3	3	Yes	No	39	2	28/338/0.8	81A	–
57, M	39	3	58/752/0.7	1	No	No	26	–	30/461/0.5	80A	–
48, F	34	5	315/180/1.1	3	Yes	Yes	8	2	30/121/2.6	3D	Graft failure
63, M	18	4	89/318/0.8	1	No	No	10	–	33/218/0.6	73A	–
45, M	30	5	192/143/2.9	2	Yes	Yes	12	1	44/180/3.6	22D	Graft failure
49, M	16	3	185/269/1.9	2	Yes	No	40	1	30/258/3.1	3D	Graft failure
47, M	9	3	44/134/1.4	1	No	No	12	–	14/118/0.7	58A	–
47, M	9	3	45/229/14.5	2	Yes	No	41	1	34/470/9.2	2D	Graft failure
49, M	12	4	127/288/0.9	1	No	No	17	1	35/225/0.5	50A	–
58, F	19	3	45/469/4.5	1	No	No	38	1	35/431/3.1	9D	TMA
Basiliximab group											
55, M	44	3	125/888/0.5	0	No	No	7	–	14/432/0.5	27A	–
61, F	49	4	112/1077/2.1	0	Yes	No	15	2	48/653/1.6	8D	Pneumonia
60, F	19	3	100/448/3.5	0	No	No	6	–	36/265/2.2	3A	–

Abbreviations: ACR, acute cellular rejection; A, alive; D, dead; TMA, thrombomicroangiopathy; MMF, mycophenolate mofetil; ALT/ALP/TB, alanine aminotransferase (IU/L)/alkaline phosphatase (IU/L)/total bilirubin (md/dL).

several reports of cytomegalovirus viremia after induction with anti-IL-2 receptor antibodies, but to date there is no evidence of a significant increased risk of cytomegalovirus infection (*12*). In this series, one patient died of pneumonia 8 months after liver transplantation, which may not be directly attributable to basiliximab therapy.

HCV recurrence developed in most HCV-positive recipients. The intensity of immunosuppression correlates with recurrent HCV hepatitis after transplantation (*13*). Importantly, Kato *et al*. (*14*) reported that tacrolimus along with daclizumab and a steroid-free regimen resulted in fewer HCV infection recurrences after transplantation. The use of basiliximab therefore seems to be advantageous over steroid therapy in HCV-positive patients.

Our results are comparable to those of other case series in which basiliximab was used as rescue therapy. Orr *et al*. (*15*) used basiliximab in 16 patients with steroid-resistant ACR, and resolution was achieved in 12 (75%) cases. Similarly, in another study (*16*), 5 (71.4%) of 7 liver transplant recipients with steroid-resistant ACR were successfully treated with basiliximab. Another series (*6*) showed resolution of ACR in adult renal and liver recipients, 72% of whom were successfully treated with basiliximab. The findings of that study (*6*) suggest that the efficacy of anti-IL-2 receptor antibody in ACR is similar to that of high-dose steroids, although there are no prospective randomized trials that have tested this hypothesis.

Anti-IL-2 receptor antibodies are now widely used in liver transplantation as induction agents targeted at reducing the incidence of ACR (*2,17,18*) or as a calcineurin-inhibitor (*19*) or steroid-sparing strategy (*20,21*) to protect renal function in the immediate postoperative period. Comparison of induction therapy (*12*) between antithymocyte globulin + cyclosporine + steroid + azathioprine and anti-IL-2 receptor antibody + tacrolimus + steroid + azathioprine demonstrated a reduced ACR incidence in the anti-IL-2 receptor antibody group (43% *versus* 29%; $p < 0.01$). Patients treated with anti-IL-2 receptor antibodies also had fewer infective complications. The results of a meta-analysis of treatment with anti-IL-2 receptor antibodies in renal transplantation (*22*) confirmed that the risk for ACR was reduced by 49% at 6 months. Importantly, however, their use did not significantly reduce graft loss or mortality at 1 year. Prospective randomized studies comparing calcineurin inhibitors + steroid + basiliximab and calcineurin inhibitors + steroid showed a lower (*23*) or similar (*24,25*) ACR rate and no difference in graft survival.

A limitation of this study is that it was a retrospective and nonrandomized case series. The small number of patients enrolled in the study was insufficient to yield statistically meaningful results. These preliminary data, however, indicate that the use of anti-IL-2 receptor antibodies is safe for the treatment of ACR in HCV-positive patients. Prospective studies are now needed to evaluate the use of anti-IL-2 receptor antibodies as a first-line therapy for ACR for HCV-positive patients.

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Original Article

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Content analysis of school textbooks on health topics: A systematic review

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Summary **High-quality textbooks and learning materials are especially important for school children, but school textbooks may contain incorrect health information. The objective of this study was to review the findings of analytical studies about the contents of textbooks used in elementary, junior high, or high schools. Of 450 studies we screened, we reviewed 14 that met the inclusion criteria, and summarized information regarding: *i*) authors and publication year, *ii*) target country, *iii*) topics selected, *iv*) school level, *v*) textbook subject(s), *vi*) analytical methods, and *vii*) findings. Of the selected 14 studies, 9 were conducted in the United States and Spain. Health topics focused mainly on sexuality, HIV/AIDS, and nutrition. The reviewed studies were classified according to the amount of topic information they contained, the accuracy of the health information provided, and the health information priorities conveyed. The findings of reviewed studies can be summarized as follows: some current school textbooks provide insufficient content and contain inaccurate or out-of-date health information. This study found through health-related content analysis of the school textbooks that textbooks in the United States and Spain cover sexuality, sexually transmitted diseases, and nutrition more often than do textbooks in other countries. Content quality is sometimes inappropriate and requires improvement.**

Keywords: School textbooks, school health, health education, content analysis, systematic review

1. Introduction

School health education has proven to be effective in increasing knowledge and improving attitudes, beliefs, and skills needed to practice healthy behaviors (*1*,*2*). School textbooks are essential materials for school health education (*3*,*4*). Particularly in resource-limited settings, school textbooks can play an important role as a source of reliable information (*5*). Because school textbooks can provide health information on disease prevention and essential health skills, the information they contain must be reliable. The United Nations Educational, Scientific and Cultural Organization (UNESCO) emphasizes improving the quality of textbooks as one of its policy recommendations within the Education For All Framework (*6*).

Despite the importance of accuracy, however, previous studies have indicated that school textbooks contain incorrect or insufficient health information (*7*). To reduce the likelihood of students receiving and accepting incorrect information, regular revision and regular improvement of content quality is essential for raising the health levels of students and their family members. Reviews of school textbooks by government authorities and the inclusion of priorities of national health policies are also recommended, yet few studies have assessed textbooks in terms of their health information content or examined their accuracy and frequency of revision.

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The objective of this study was to review the findings of analytical studies of the contents of textbooks used in elementary, junior high, and high schools.

2. Methods

2.1. *Search strategy and inclusion/exclusion criteria*

We did a search for eligible literature regarding school health and school textbooks. First, we conducted an electronic search of popular academic databases for health and education. Our electronic search strategy was first to look at the PubMed and the Education Resources Information Center (ERIC) Internet databases. PubMed is a service of the U.S. National Library of Medicine from MEDLINE and other life science journals of a biomedical nature. ERIC is an online digital library of education research and information, sponsored by the Institute of Education Sciences of the U.S. Department of Education. Both databases seemed appropriate choices because they are widely used in their respective fields (medicine and education) and cover the key words of the reviewed studies. In both PubMed and ERIC, the key words "schools" AND "textbooks" AND "health" were used to retrieve articles published between January 1980 and June 2009, with no language restrictions. The search strategy also included a review of the references cited by the identified studies. The process and the number of systematic reviews are shown in Figure 1.

The inclusion criteria were the following: the study must include content analysis of school textbooks; the textbooks examined must be used in elementary, junior high, or high school; the textbooks must include health-related information; and the articles must have been published between 1980 and 2009. As education systems in different countries differ, school levels were classified according to grades or ages, and defined as elementary school, junior high school, or high school. Content analysis is defined as the systematic, objective, qualitative analysis of message characteristics (*8*). Exclusion criteria were content analysis of content unrelated to health, use of intervention analysis, or use of the textbooks in kindergartens or college/university-level education.

Titles and abstracts of studies were screened primarily using the key words. Two reviewers independently assessed all titles and abstracts retrieved through the electronic searches. An initial relevance screening generated 450 studies in total from the literature search, including 245 studies from PubMed

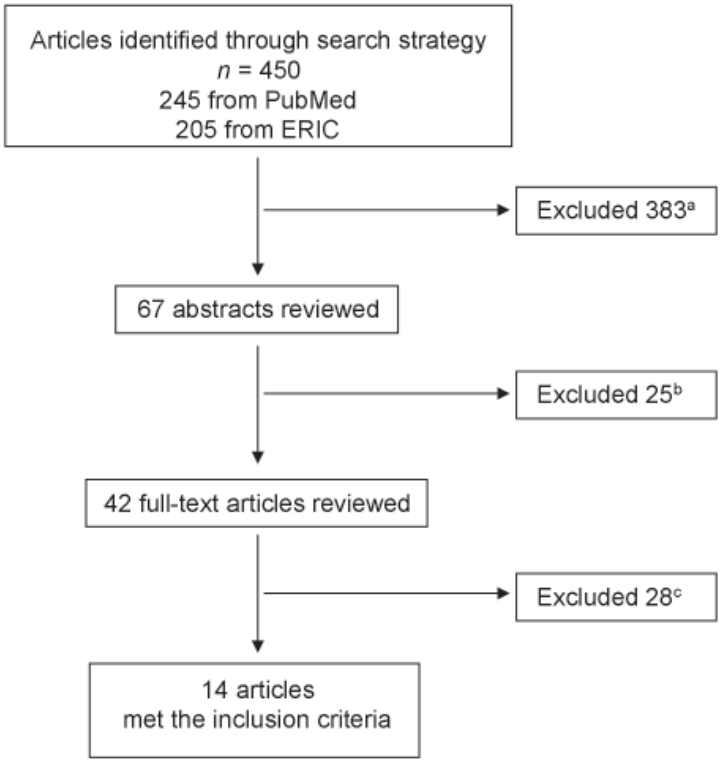

Figure 1. Flow chart of the study selection process. [a] The 383 excluded articles included those with no content analysis or content analysis on other topics. [b] These 25 articles were excluded because target school levels did not meet the inclusion criteria. [c] These 28 excluded articles included intervention studies or content analysis not related to health topics.

and 205 from ERIC.

Of these 450 studies, 383 were excluded due to a lack of content analysis or a suggestion in the titles or abstracts that the content analysis was of non-health-related content. This screening yielded 67 studies that met the initial inclusion criteria. Next, the abstracts of the 67 studies were collected and independently reviewed, and excluded from full-text review if the content analysis was not focused on school textbooks or if the topic was not health-related.

Of the 67 studies, 25 were excluded because their target school levels did not meet our inclusion criteria. The remaining 42 studies returned by the searches were included in the full-text review. Full-text copies of the 42 studies identified as potentially relevant were retrieved; their full texts in English were collected and reviewed; they included 8 non-English studies that had been translated into English. In cases where reviewers disagreed on the eligibility of reviews, a discussion was held in order to obtain consensus. The studies in full-text were included if they met all of the above-mentioned inclusion criteria (use of content analysis, use of textbooks in elementary, junior high, or high school, and focus on health information, including hygiene, infectious diseases, and other health topics). We excluded intervention studies and systematic reviews. The 42 full-text screened studies were reexamined several times by two reviewers.

Of the 42 full-text studies, 28 were excluded because they included intervention studies or systematic reviews, or they analyzed non-health content. Ultimately 14 studies met the inclusion criteria and were examined. Of the 14 studies, 5 were not written in English: 3 were in Spanish (*4*,*9*,*10*), 1 was in Portuguese (*11*), and 1 was in German (*12*). We asked native speakers of Spanish, Portuguese, and German to translate the 5 studies into English and reviewed the English translations. Searches were conducted between May and August 2009.

2.2. *Assessment*

All the studies of content analysis were examined from two aspects: the content of relevant domains, and the total numbers of pages, tables, figures, and pictures/illustrations. All were summarized using a form containing 7 categories for comparison. The 7 categories included: *i*) authors of references and year of publication, *ii*) target country, *iii*) specific health topic, *iv*) school level or grade, *v*) textbook subject(s), *vi*) analytical method(s), and *vii*) findings. This process resulted in a document with tables.

3. Results

We summarized the characteristics of the 14 studies of content analysis we examined in Table 1.

3.1. *Target country*

Of 14 studies, 12 were conducted in North America or Europe. The target countries were: the United States in 6 studies, Spain in 3, and Brazil, Canada, Mexico, Switzerland, and the United Kingdom in 1 each. Twelve of the studies were conducted in developed countries, 2 in middle-income countries, none in low-income countries.

3.2. *Specific health topics covered*

Of the 14 studies, 2 evaluated the priorities of the health information content in the textbooks (*4*,*9*). The main health topics in 12 studies were sexuality, sexually transmitted diseases (STDs), and nutrition. Of the included studies, 4 focused on sexuality, including reproductive health, gender representation, and STDs (*7*,*13-15*), 3 focused on nutrition or diet, including excessive intake of sugar-rich food (*12*,*16*,*17*), and 1 each focused on HIV/AIDS (*10*), mental health (*18*), hearing health (*19*), oral cancer (*20*), and vaccination (*11*). Two other Spanish studies identified the priorities of health topics covered. In a study conducted by Catalán in 2003, the priorities were hygiene, followed by eating (*9*); in the other study conducted by Barrio Cantalejo *et al*. in 2008, they were diet, physical exercise, and the impact of environmental contamination (*4*).

3.3. *Target school levels and textbook subjects*

Target school levels and textbook subjects were diverse. Of the 14 studies, 9 studies targeted a single school level: 5 targeted elementary schools, 3 targeted junior high schools, and 1 targeted high schools. The remaining 5 targeted multiple school levels: 1 study each targeted elementary and junior high schools, elementary and high schools, or junior high and high schools; the remaining 2 studies targeted all three school levels. The textbooks examined covered the full range of subjects: health science, language, arts, mathematics, history, science, arithmetic, algebra, and chemistry.

3.4. *Analytical methods*

All 14 studies examined were descriptive studies of content analysis. Content analysis is an in-depth analysis of messages using quantitative or qualitative techniques. The studies' analytical methods fell into three main patterns: *i*) analysis of the amount of topic information in words, pictures, and/or illustrations (8 studies); *ii*) examination of the accuracy of health information (2 studies); *iii*) identification of health-related topics and their priorities (2 studies). Of the two remaining studies that did not fall into any of the three main patterns, one identified the impact of political and

Table 1. Results of reviews by target country, school grade level, subjects, and methodology

Author, year	Target country	Specific health topics	School level and grades	Subjects of texts	Analytical methods	Findings
Tolan and Lounsbury, 1982	US	Mental health	High school. No information about grades.	Health textbooks	Examination of community mental health ideology presented in high school health textbooks.	Information in high school health textbooks does not adequately represent modern mental health knowledge or practice. It does not meet society's mental health needs.
D'Onofrio and Singer, 1983	US	Nutrition, sugar, sweets	Grades K-3 in elementary school.	Readers, pre-readers, and reading workbooks	Analysis of food-related content in words and pictures in texts.	Food-related content in the texts revealed an excessive emphasis on sweets in both words and pictures. The poor results of nutrition education have been publically decried. Unintended messages may work against the promotion of healthy eating habits. Textbook revision is clearly indicated.
Kroger and Yarber, 1984	US	STD and sexuality	Junior high school. No information about grades.	Health science and sex education	Assessment of potential contribution of textbooks to STD control objectives.	Sex education textbooks contribute to STD control objectives better than do health science textbooks. Health science textbooks may not contribute toward reducing STD incidence. Both types of textbooks present biomedical information without significant errors.
Frager and Kahn, 1988	US	Hearing health and protection	Elementary school. No information about grades.	School health textbooks	Identification of health information on hearing and assessment of its usefulness to prevent hearing loss.	More content regarding the signs and causes for hearing problems was identified than recommendations for avoiding hearing problems. Hearing health mobilizing information is lacking.
Beyer *et al.*, 1996	US	Gender representation	Junior high and high school. No information about grades.	History, mathematics, science, and reading	Examination of gender inequity in written textbooks.	Illustrations showed greater female representation. Greater male representation included that related to drug use, sexual exploitation, sexual desire, and homosexuality. Greater female representation included that related to body image, diseases of the reproductive organs, and hygiene.
Baysac *et al.*, 2004	US	Oral cancer	Grades 1-12 in elementary, junior high, and high school.	Health education	Evaluation of quality, completeness, and accuracy of oral cancer information.	Current school health textbooks do not provide adequate information about oral cancer prevention and early detection. To achieve Healthy People 2010 objectives, correct and adequate information about risk factors and examinations for oral cancer are needed.
Gavidia Catalán, 2003	Spain	All health information	Elementary, junior high, and high school. No information about grades.	All subjects	Identification of 1) presence or absence of education for health, 2) inclusion of health-related topics, 3) degree of health covered, 4) inclusion in the subject or dealt with on a transversal basis, 5) methodological aspects.	Sixty-three percent of the texts analyzed included topics on health education. Most topics were related to hygiene and eating. Health-related concepts most often dealt with are those of being disease-free and in a state of well-being. Current school textbooks are not sufficient either as a point of reference or as an adequate resource.
Barrio Cantalejo *et al.*, 2008	Spain	All health information	Elementary and high school. No information about grades.	No information about subjects.	Identification of 1) health priorities defined by health organizations, 2) messages on health, 3) extent to which these messages fit the priorities established.	The priorities most frequently covered in the textbooks were diet, physical exercise, and the impact of environmental contamination. The health messages contained in school textbooks are not well adapted to the priorities defined by health organizations.

Abbreviations: STD, sexually transmitted diseases; STI, sexually transmitted infection.

(*to be continued*)

Table 1. Results of reviews by target country, school grade level, subjects, and methodology (*continued*)

Author, year	Target country	Specific health topics	School level and grades	Subjects of texts	Analytical methods	Findings
de Irala *et al.*, 2008	Spain	Sexuality, human reproduction, and STIs	14-15-year-olds in secondary school	Biology	Evaluation of the extent to which textbooks on sexuality and human reproduction promote healthy reproductive lifestyles as well as avoidance of risk behavior among adolescent students.	All textbooks presented inaccurate information and incomplete perception of sexuality or risky behavior. On average, 12.6 incorrect messages were identified in each textbook. Eleven of 12 textbooks examined provided misleading statements on condom use for contraception and STI prevention and on family planning methods. The textbooks were neither appropriate nor sufficiently comprehensive for adolescent education on issues of sexuality. Results suggest a need for alternative textbooks based on better scientific evidence. Teenage sexual activities described in the textbooks are not supported by epidemiological data from the Spanish National Institute of Statistics.
Succi *et al.*, 2005	Brazil	Vaccines	Grade 1-8 in elementary school	Science and biology	Evaluation of content of textbooks with regard to concepts and information on vaccination.	Despite Ministry of Education recommendations, 34% of elementary-level textbooks did not include the subject of vaccination. More than half of the textbooks with content on vaccines presented some erroneous information on vaccination, errors in vaccination schedules, out-of-date information, omission of content, or inadequate illustrations.
Baron, 1990	Canada	Nutrition	Grade 1-6 in elementary school	Language, arts, and mathematics	Detection of nutrition messages in words and pictures.	A large proportion of references were to sugar-rich foods. Unintended information may influence nutritional habits of children.
Granados -Cosme *et al.*, 2007	Mexico	HIV/AIDS prevention	Grade 5-6 in elementary and 1-3 grade in junior high school	Natural science, biology, civics, and ethics education	Clarification of social actors' positions and interests and their influence on the content of textbooks.	Those actors whose beliefs are based on tradition and are contrary to modernization oppose the inclusion of topics on sexuality and HIV/AIDS in the school curriculum. The deficiencies and decline in HIV/AIDS prevention education were caused by actions from opposition groups.
Eichholzer -Helbling *et al.*, 1984	Switzer-land	Nutrition	Grade 1-4 in elementary school	Reading, arithmetic, and language	Examination of contents regarding nutrition in the textbooks.	Educational information regarding nutrition can be found in all textbooks, but it was not adjusted to today's perceptions.
Reiss, 1998	UK	Sexuality	14-15-year-olds in high school	Biology and science	Analysis of health topics related to human sexuality in school science textbooks.	Some science textbooks are sensitively written, comprehensive, and helpful. Others fail to tackle personal issues dealing with menstruation, ignore lesbian and gay issues, and either omit or fail to deal adequately with cultural issues in spite of the regulations of the UK Government's own Circular.

Abbreviations: STDs, sexually transmitted diseases; STI, sexually transmitted infection.

social ideology on the content of HIV/AIDS education (*10*), another identified gender representation and examined gender inequity in textbook descriptions (*14*).

As the objective basis of analyses, 4 studies cited the country's national health policies or recommendations as a standard, and compared them with the content of health information in the textbooks. The 4 studies cited Healthy People 2010 in the United States, health priorities defined by health organizations or authorities in Spain, or recommendations of the Ministry of Education in Brazil. Other studies had no standards by which to evaluate the contents.

3.5. *Findings*

To summarize the quality of the school textbooks examined in findings, we chose the amount, the accuracy, and the currency of the health information on target health topics. We showed the results using the "+" symbol in Table 2. Of 14 studies reviewed, 11 reported that the school textbooks examined provided insufficient content or lacked information regarding the target topics; 5 studies reported that the health information in textbooks included inaccuracies or false information; and 5 studies reported that the health information was not current or was out-of-date and needed revision. In total, the authors of 13 of the 14 studies indicated that the textbooks they examined needed further improvement or revision.

In comparisons of the contents with national health policies or priorities, 4 studies cited the target country's health policy or Ministry of Health/ Education recommendations as standards. For example, they cited Healthy People 2010 objectives (*20*), the guidelines of Ministry of Education (MEC) (*11*), the UK Government's own Circular, the Local Government Act, and the Education Act (*15*), 24 priorities defined by the World Health Organization, the European Union, the Spanish Ministry of Health and Consumer Affairs, and the Spanish Society of Public Healthcare Administration (*4*).

4. Discussion

Content analysis of school textbooks often focused on sexuality/reproductive health and STDs; 4 out of 14 studies focused on these topics. These topics are important because unintended pregnancy is one of the main reasons why female students drop out of school (*21*,*22*). Furthermore, young people are particularly vulnerable to HIV infection: 15-24-year-olds account for 50% of new cases worldwide (*23*). They must be provided with essential skills and information before they become sexually active (*24*). The authors of these studies examined textbooks used in elementary and junior high schools (targeting 14-15-year-old students).

Although sex education is known to be difficult to deliver in school settings (*25*), previous studies have reported that school children in many countries identify textbooks or school as their primary source of health information (*26-29*). The importance of information delivered in school settings has also been demonstrated by its long-term impact on healthy behaviors (*30*,*31*). Thus, the contents of school textbooks require regular revision to provide students with accurate health information regarding sexuality, STDs, and reproductive health.

Three studies highlighted the over-representation of graphical information presenting sugar-rich food in the textbooks (*12*,*16*,*17*). The authors suggested the potential impact on school children of the over-representation of unhealthy eating behaviors. As

Table 2. Quality of the school textbooks examined

Author, year	Specific health topics	Quality		
		Insufficient information	Lack accuracy	Out-of-date information/ need revision
Tolan and Lounsbury, 1982	Mental health	+	+	+
D'Onofrio and Singer, 1983	Nutrition		+	
Kroger and Yarber, 1984	STD and sexuality	+		
Frager and Kahn, 1988	Hearing health and protection	+		+
Beyer *et al.*, 1996	Gender representation	+		
Baysac *et al.*, 2004	Oral cancer	+	+	
Gavidia Catalán, 2003	All health topics	+		
Barrio Cantalejo *et al.*, 2008	All health topics	+		
de Irala *et al.*, 2008	Sexuality, human reproduction, and STIs	+	+	+
Succi *et al.*, 2005	Vaccines	+	+	+
Baron, 1990	Nutrition			
Granados-Cosme *et al.*, 2007	HIV/AIDS prevention	+		
Eichholzer-Helbling *et al.*, 1984	Nutrition			+
Reiss, 1998	Sexuality	+		

+: include the comment shown at the top of the column.

unintended messages conveyed in textbooks may counteract efforts to promote healthy eating habits, those responsible for textbook selection should draw up health content guidelines.

In the 14 studies we reviewed, none focused on tobacco use, injury prevention, or alcohol/substance abuse, which have been emphasized as central to health education curricula in school settings (*32*,*33*). Our results also revealed that the researchers paid little attention to these topics in content analysis. However, educational interventions using various approaches including peer-education and linkage with supportive communities and policies have been intensively utilized in the school setting (*34*-*36*). Nonetheless, these studies emphasized the importance of comprehensive approaches and of basic curricula as principal components of health education.

The findings examined by the studies we reviewed were summarized as follows: most of the reviewed articles consistently reported insufficient health information provided by the textbooks. This tendency was particularly evident in the studies dealing with sexuality or STDs (*7*,*13*,*15*). However, 13 out of 14 studies reviewed also reported a wide range of variation among publishers in content insufficiency, inaccuracy, or out-of-date information.

In comparisons of content with national health policies or recommendations, only 4 studies cited the country's national health policy or recommendations as a standard. To evaluate the quality of school textbooks objectively, some standard of comparison is needed.

We also found that studies were mostly confined to developed nations: the target countries of 13 of the 14 studies were the U.S., Spain, Canada, Switzerland or the United Kingdom. This might be due to wider availability of textbooks in developed countries. However, 80% of the world's children live in developing countries where resources for textbook development are more likely to be limited than in developed countries (*37*,*38*). School textbooks used in developing countries also need to be examined with a view to improving the contents of health information.

To regularly and reliably provide sufficient and accurate health information in school textbooks to school children, we need to assess the adequacy of all school textbooks, whether they are used in developed or in developing countries.

5. Conclusion

In conclusion, this study showed that health-related content analysis of school textbooks is done mostly in Spain and the United States and most frequently examines content related to sexuality, STDs, and nutrition. The quality of the content is sometimes inappropriate and requires improvement.

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Medical students' choice of specialty and factors determining their choice: A cross-sectional questionnaire survey in Melaka-Manipal Medical College, Malaysia

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Summary **Information about medical students' choice of specialty can be helpful for planning health manpower. However, such information from medical students in Malaysian medical schools is lacking. We carried out a cross-sectional questionnaire survey among fourth- and fifth-year medical undergraduate students at Melaka-Manipal Medical College. A total of 425 students responded to the survey questionnaire. Nearly a quarter of the students indicated internal medicine as their choice of specialty. Other choices were general surgery (13.2%), pediatrics (11.3%), orthopedics (12.7%) and obstetrics & gynecology (Ob/Gyn) (12.1%). Female students (OR 1.91; 95% CI 1.18-3.08), fourth-year students (OR 1.9; 95% CI 1.15-3.12), and students who reported a higher self-rated knowledge of their subject of choice were more likely to choose internal medicine and allied specialties (OR 1.53; 95% CI 1.07-2.19). The influence of teaching faculty and consultants at the teaching hospitals (74.4%) and inspiration obtained during clinical postings (71.9%) were the factors which were rated by the most students as 'important' for choosing a specialty. About half of the students intended to pursue their postgraduate studies in Malaysia, most of the rest in the United Kingdom or Australia. While internal medicine and surgical subspecialties were preferred, students were not inclined towards primary care or diagnostic subspecialties. Incentives should be provided and other measures should be taken to make these branches more attractive.**

Keywords: Undergraduate medical education, postgraduate training, specialty, Malaysia

1. Introduction

Medical education requires undergraduate students to study a wide range of medical subjects. Medical careers begin undifferentiated; during their postgraduate training, doctors specialize in a particular field of practice. Medical students can be seen as a relatively undifferentiated, multi-potent 'stem doctors' (*1*) capable of entering any field of practice; specialization turns them ultimately into fully-differentiated specialists whose practice is almost entirely confined to one specialized branch of medicine. Information about students' preferred choice of specialist training may be useful in planning national health manpower programs and in identifying the specialties with low preference in order to undertake measures to make these specialties more attractive. Such information may also help medical educators to plan training facilities and define selection criteria.

Medical students' decisions about specialist training may be influenced by several factors. However, very little is known about the factors that influence medical students in South and Southeast Asia. Based on the

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existing literature, these factors are thought to include higher income, prestige, hospital-based practice (*2*), experience during clinical postings, inspiration given by academic teaching faculty, working hours, and flexibility of working arrangements (*3*,*4*). Personal intelligence and career opportunities also play a role (*4*). Besides the afore-mentioned factors, advice from family, friends, or practicing specialists may also influence students in deciding their future specialty. In addition, personal life experiences, interest in community-based settings, and passion for providing continuous care to patients may also pave the way to a specialty choice.

Several studies from different countries have reported preferred choices of medical specialist training and factors affecting medical students' choice of specialty. A study from the United States of America (USA) reported that no single factor dominates a student's choice for primary care, and higher income, more prestige, and hospital-based practice play an important role in the choice of non-primary care specialties (*2*). Work culture, working conditions, and personal experience with discipline-based medicine in the early postgraduate years were reported to be important factors among Australian undergraduate medical students (*3*). In New Zealand (*5*), medicine, surgery, general practice (GP), pediatrics, and obstetrics & gynecology (Ob/Gyn). were the most popular choices and faculty and clinical posting experiences were reported as the main influencing factors. A study from Canada (*6*) compared family medicine with other specialties and found that older students, those concerned with medical lifestyles, those who were living in smaller communities during high school, and those more interested in varied-scope practice and less interested in hospital-oriented practice preferred family medicine to other specialties. Personal intelligence, ability preferences, and career opportunities were important factors among Chinese medical undergraduate students (*7*). A similar study from Jordan (*8*) reported that surgery, Ob/Gyn, internal medicine, and pediatrics were preferred specialties, and the specialty's reputation, anticipated income, focus on urgent care, intellectual content of the specialty, and individual's competency were the factors most influencing their choice. Preclinical and clinical experiences as well as role models were the factors reported from Japan (*4*). Prestige, money, and personal development were reported as important factors among Turkish undergraduate medical students (*9*). However, there are no such data about choice of specialty and factors influencing choice among Malaysian medical students. The objectives of our survey were to identify medical students' choice of specialty and the factors considered most important by medical students in Malaysia when choosing their specialty.

2. Methods

2.1. *Study design and setting*

The study was designed as a cross-sectional self-administered questionnaire-based survey. In Malaysia, undergraduate medical education is offered by both publicly-funded and private medical schools. The undergraduate medical program is of five years' duration with compulsory rotational housemanship after completion of the final qualifying examination. The curricula in most medical schools incorporate problem-based learning (PBL), with more emphasis on tutorials than lectures. In most medical schools, clinical rotations start from the third year. Students undergo clinical training in major medical, surgical, and allied subjects during each academic year, including senior clerkships during the fifth year. Postgraduate specialist training facilities are mostly available in publicly-funded university medical schools; recently, a few private medical schools have also initiated postgraduate specialist training.

This survey was carried out among the undergraduate medical students of Melaka-Manipal Medical College (MMMC), Melaka Campus, Malaysia. MMMC is a private medical college which offers a twinning medical course and is affiliated with Manipal University, India. Students of MMMC undergo 2.5 years of basic sciences and introductory clinical training on the Manipal Campus in India. Subsequently, another 2.5 years of clinical training takes place at the Melaka Campus in Malaysia. The medical degree Bachelor of Medicine and Bachelor of Surgery (MBBS) conferred by Manipal University is recognized by the Government of Malaysia and the Malaysian Medical Council (MMC) and is accredited by the Malaysian Qualifications Agency (MQA). MMMC is also listed in the WHO directory of recognized medical colleges and in the International Medical Education Directory (IMED) of the Educational Commission for Foreign Medical Graduates (ECFMG).

2.2. *Participant*

All fourth- and fifth-year undergraduate medical students studying at Melaka-Manipal Medical College, Melaka campus, were eligible to participate in the survey. These students were chosen because they had already completed at least one clinical rotation in all clinical subjects.

2.3. *Questionnaire*

After a detailed review of existing literature and informal discussions with medical students and teaching faculty at MMMC, a structured questionnaire was developed in English. A draft questionnaire

was subjected to a validation process in which the questionnaire was given to a group of 15 medical students to obtain their feedback and check for item appropriateness and comprehensiveness. The questionnaire was revised based on the responses and feedback received. The questionnaire began with instructions to the participants followed by demographic data. The questions regarding the choice of subject in which the students intended to specialize were open-ended. The participants were instructed to identify their three most preferred subjects, *i.e.*, their first, second, and third choices. A list based on the review of literature of 16 reasons for and factors associated with the students' choice of specialty was also included. The participants were asked to rate the importance of these reasons and factors as 'least important' to 'most important' on a five-point Likert scale. The questionnaire also contained a few questions regarding their self-rated knowledge on their preferred subject choice on a five-point Likert scale and regarding the country where they intended to pursue their postgraduate training.

2.4. *Data collection*

Permission/approval to carry out the survey was obtained from college authorities and the Research Committee. During August and September 2010, data collection was done by members of our team, namely Chew Yu Wei (CYW), Sudeash Rajakrishnan (SR), Low Chin Aun (LCA), and Prakash Kumar Jayapalan (PKJ). Structured questionnaires were distributed to the students before or after small group teaching sessions. All the students in their fourth or fifth years were invited to participate in the survey after being briefed about the objectives. The students were also informed that participation in the survey was voluntary and not compulsory. Assurances of anonymity and confidentiality of the information were given. Consent was sought from all the students before the questionnaires were administered. The students were not required to enter any identifiable personal details like name, address, or roll number on the questionnaire. Completed questionnaires were collected by the research team.

2.5. *Variables*

The 16 possible reasons and factors for the student's choice of specialty were grouped into 'personal' and 'professional' reasons/factors. We computed separate scores for personal reasons and for professional reasons by adding the Likert scores. We used first choice of specialty in our multivariate analysis to identify factors determining the choice of internal medicine and allied subjects (*i.e.*, internal medicine, pediatrics, cardiology, neurology, emergency medicine, primary care medicine, *etc*.). The other category was surgical branches or specialties, including general surgery, orthopedics, obstetrics, gynecology, otolaryngology, *etc*. The choice of internal medicine and allied subjects was used as a dependant variable. We used demographic variables, year of study, self-rated knowledge about their first choice, and reasons/factors influencing the choice (Likert scores for personal reasons and for professional reasons) as independent variables for univariate and multivariate analyses.

2.6. *Statistical analysis*

Statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) version 14. We generated summary statistics for demographic variables and choice of medical specialty. We carried out univariate and multivariate analyses to assess the factors determining the choice of internal medicine and allied subjects. We calculated crude and adjusted odds ratios (OR) and their 95% confidence intervals (95% CI). A p value less than 0.05 was considered significant.

3. Results

3.1. *Response rates and demographic characteristics*

Out of 524 students in their fourth- or fifth-year MBBS program, 425 students responded to the questionnaire, giving an overall response rate of 81.2%. Of these students, 23 had not completed all the sections of the questionnaires and were not included in the analysis. Thus 402 completed questionnaires were analyzed. Of these students, 243 were in their fourth year and 159 were in their fifth year. The students' median age was 23 years (mean = 23.6 years, S.D. = 1.02). Demographic characteristics of the respondents according to gender are shown in Table 1. About 73% of the students were single, 26% were currently in a relationship. The ethnic distribution of students was nearly a third each for Malay, Chinese and Indian. The vast majority (97%) of the students were Malaysians; 12 students were from Seychelles or Sri Lanka. About 9% of the students had a medical doctor parent; 40% had one or more siblings who were doctors.

3.2. *Choice of medical specialty*

Thirty-one students (7.7%) did not indicate any intention to specialize in any subject. For nearly one-fourth of the students, internal medicine was their first, second, as well as third choice. General surgery (13.2%), pediatrics (11.3%), orthopedics (12.7%) and Ob/Gyn (12.1%) were indicated as first choice by almost equal percentages of students (Table 2). Men were more likely to choose surgical branches while women were more likely to choose internal medicine

Table 1. Socio-demographic characteristics of participants

	Male (*n* = 177)	Female (*n* = 225)	Total (*n* = 402)	*p*
Nationality				
Malaysian	173 (97.7%)	217 (96.4%)	390 (97.0%)	
Non-Malaysian	4 (2.3%)	8 (3.6%)	12 (3.0%)	0.448
State of residence				
West Malaysia				
North	47 (27.2%)	71 (32.7%)	118 (30.2%)	
South	115 (66.5%)	136 (62.7%)	251 (64.4%)	
East Malaysia	11 (6.4%)	10 (4.6%)	21 (5.4%)	0.421
Ethnicity				
Malay	46 (26.0%)	79 (35.1%)	125 (31.1%)	
Indian	56 (31.6%)	75 (33.3%)	131 (32.6%)	
Chinese	69 (39.0%)	62 (27.6%)	131 (32.6%)	
Others	6 (3.4%)	9 (4.0%)	15 (3.7%)	0.078
Religion				
Islam	49 (27.7%)	87 (38.7%)	136 (33.8%)	
Buddhist	47 (26.6%)	50 (22.2%)	97 (24.1%)	
Christian	25 (14.1%)	23 (10.2%)	48 (11.9%)	
Hindu	43 (24.3%)	55 (24.4%)	98 (24.4%)	
Others	13 (7.3%)	10 (4.5%)	23 (5.8%)	0.135
Year of study				
Fourth	95 (53.7%)	148 (65.8%)	243 (60.4%)	
Fifth	82 (46.3%)	77 (34.2%)	159 (80.5%)	0.014
Parents' occupation				
Doctor	15 (8.5%)	22 (9.8%)	37 (9.2%)	
Others	162 (91.5%)	203 (90.2%)	365 (90.8%)	0.650
Siblings' occupation				
Doctor	66 (37.3%)	95 (42.2%)	161 (40.0%)	
Others	111 (62.7%)	130 (57.8%)	241 (60.0%)	0.316

Table 2. Specialty preferences among fourth- and fifth-year medical students

Subject of choice for specialization	First choice (*n* = 371)	Second choice (*n* = 333)	Third choice (*n* = 259)	Total (*n* = 963)
Medicine	97 (26.1%)	75 (22.5%)	57 (22.0%)	229 (23.8%)
Surgery	49 (13.2%)	61 (18.3%)	29 (11.3%)	139 (14.4%)
Pediatrics	42 (11.3%)	22 (6.6%)	20 (7.7%)	84 (8.7%)
Ob/Gyn	45 (12.1%)	39 (11.7%)	32 (12.4%)	116 (12.0%)
Orthopedics	47 (12.7%)	28 (8.4%)	26 (10.0%)	101 (10.5%)
Otolaryngology	16 (4.4%)	33 (9.9%)	14 (5.4%)	63 (6.5%)
Ophthalmology	7 (1.9%)	14 (4.2%)	15 (5.8%)	36 (3.8%)
Psychiatry	12 (3.2%)	7 (2.1%)	15 (5.8%)	34 (3.5%)
Public Health	12 (3.2%)	11 (3.3%)	8 (3.1%)	31 (3.2%)
Emergency Medicine	11 (3.0%)	13 (3.9%)	11 (4.2%)	35 (3.7%)
Anesthesia	14 (3.8%)	12 (3.7%)	10 (3.9%)	36 (3.8%)
Radiology	9 (2.4%)	11 (3.3%)	11 (4.2%)	31 (3.2%)
Others	10 (2.7%)	7 (2.1%)	11 (4. 2%)	28 (2.9%)

subjects. Ob/Gyn was indicated by 37 (16.4%) female students and by only eight (4.5%) male students. Nearly 50% of the students wanted to undergo specialty training in Malaysia, about 45% in the United Kingdom, followed in popularity by Australia.

3.3. *Factors affecting choice of medicine and allied subjects for specialist training*

By univariate analysis (Table 3), the proportion of female students choosing medical subjects was significantly higher than that of males (39% *versus* 26%, OR 1.83, 95% CI 1.19-2.81). Fourth-year students were 1.56 times more likely to choose an internal medicine specialty than fifth-year students (39.4% *versus* 29.3%, OR 1.56, 95% CI 1.03-2.38). Students with a higher self-reported knowledge about their subject of choice were 1.81 times more likely to select that subject (OR 1.81, 95% CI 1.25-2.64). Students who rated personal factors as 'important' or 'very important' were 1.06 times more likely to choose a medical subject ($p = 0.01$, 95% CI 1.02-1.12). Similarly the professional factors score also differed significantly (OR 1.06, 95% CI 1.01-1.10). The following factors were not statistically significant: age ($p = 0.917$), marital status ($p = 0.75$), postgraduate country ($p = 0.192$), sibling doctors ($p = 0.238$).

The results of multivariate analysis are shown in

Table 3. Univariate analysis of medical subjects as student's choice of specialisation

Factor/variable	Medical specialty	Odds Ratio (95% CI)	*p*
Age (mean and S.D.)*	23.6 years	1.01 (0.82-1.24)	0.917
Gender			
Male	46/177 (26)	1	
Female	88/225 (39)	1.83 (1.19-2.81)	0.006
Ethnicity			
Indian	51/131 (38.9)	1	
Chinese	46/146 (31.5)	1.52 (0.90-2.55)	0.120
Malay	37/125 (29.6)	1.38 (0.85-2.27)	0.197
Marital status			
In a relationship	35/109 (32.1)	1	
Single	99/293 (33.8)	0.93 (0.58-1.48)	0.750
Year of study			
Year 5	63/160 (39.4)	1	
Year 4	71/242 (29.3)	1.56 (1.03-2.38)	0.037
Country for postgraduate studies			
Malaysia	61/187 (32.6)	1	
Non-Malaysia	72/184 (39.1)	1.33 (0.87-2.03)	0.192
Doctor sibling			
No sibling doctor	48/166 (30.0)	1	
At least one doctor	86/241 (35.7)	1.30 (0.84-1.99)	0.238
Knowledge score (mean and S.D.)*	2.2 (0.6)	1.81 (1.25-2.64)	0.002
Personal score (mean and S.D.)*	27.6 (4.6)	1.06 (1.02-1.12)	0.010
Professional score (mean and S.D.)*	26.0 (5.2)	1.06 (1.01-1.10)	0.090

* For these variables we compared medical subjects with 'other' subjects.

Table 4. Female students were 1.91 times more likely to choose internal medicine than males (OR 1.91, 95% CI 1.18-3.08). Fourth-year students were 1.9 times more likely to select internal medicine than fifth-year students (OR 1.9, 95% CI 1.15-3.12). The score of self-rated knowledge on subject of choice was associated with choice of subject (OR 1.53, 95% CI 1.07-2.19). Professional factors also determined the choice of an internal medicine speciality (OR 1.06, 95% CI 1.00-1.12). Other factors like age (p = 0.339), country of choice for postgraduate education (p = 0.296), and personal factors (p = 0.647) were not statistically significant.

Participants were asked to rate the importance of 16 factors that may have influenced their choice of specialty; the ratings are shown in Table 5. Among personal factors, 74.4% of the students reported the 'influence of teaching faculty and consultants at teaching hospitals' and 53.8% 'possession of competencies required for the specialty' as 'important'. Among professional factors, 71.9% students responded 'inspiration obtained during clinical postings' and 63.4% 'suitability of the specialty to their own personality' as important.

Table 4. Multivariate analysis of students' choice of specialty

Factor/variable	Odds Ratio (95% CI)	*p*
Age	1.12 (0.88-1.43)	0.339
Gender		
Male	1	
Female	1.91 (1.18-3.08)	0.008
Ethnicity		
Malay	1	
Chinese	1.77 (0.98-3.20)	0.060
Indian	1.76 (1.01-3.07)	0.045
Year of Study		
Year 4	1	
Year 5	1.89 (1.15-3.12)	0.012
Country for postgraduate training		
Malaysia	1	
Non-Malaysia	1.28 (0.81-2.04)	0.296
Knowledge	1.53 (1.07-2.19)	0.019
Personal score	1.02 (0.95-1.08)	0.647
Professional score	1.06 (1.00-1.12)	0.045

4. Discussion

Our survey results showed that internal medicine was the most preferred subject for specialty training, followed by general surgery, pediatrics, orthopedics and Ob/Gyn. Most students preferred Malaysia, the United Kingdom, or Australia for their postgraduate education. Univariate analysis showed students in their fourth year and female students to be more likely to choose internal medicine and allied subjects for their specialist training. Personal factors and students' (good) self-rated knowledge of the specialty also influenced their choice of internal medicine and allied subjects for specialist training. Multivariate analysis showed female gender, year of study (fourth year), a higher self-rated knowledge, and professional factors to be determinants of the choice of internal medicine and allied subjects for specialist training.

To the best of our knowledge, studies about medical students' choice of specialty have not been carried out in

Table 5. Reasons or factors considered by medical students when choosing a specialty

Reasons/factors	Important number (%)	Very important number (%)
Personal factors		
1. Influence of teaching faculty and hospital consultants	134 (40.2)	125 (34.2)
2. Advice from parents/siblings/relatives	76 (20.8)	34 (9.3)
3. Advice from friends/seniors	89 (24.3)	23 (6.3)
4. Possession of competency needed for this specialty	127 (34.7)	70 (19.1)
5. Financial rewards	82 (22.4)	46 (12.6)
6. Less work pressure and better quality of life	99 (27.0)	57 (15.6)
7. Less working hours/ability to spend time with family	98 (26.8)	71 (19.4)
8. Personal experience pertaining to the field	129 (35.2)	56 (15.3)
Professional factors		
9. Inspiration during clinical posting	140 (38.3)	123 (33.6)
10. Advice from practicing doctors	128 (35.0)	53 (14.5)
11. Lack of experts in the field in Malaysia	91 (24.9)	37 (10.1)
12. Continuous care for patients	138 (37.7)	47 (12.8)
13. No night calls	56 (15.3)	60 (16.4)
14. Preference for community-based settings	80 (21.9)	30 (8.2)
15. Suitability of specialty to own personality	149 (40.7)	83 (22.7)
16. Very challenging nature of this field	145 (39.6)	75 (20.5)

There are missing values, because in each case, a few students did not respond.

Malaysia or South and Southeast Asia. Our results can help medical educationists and policy makers to plan postgraduate training and health manpower programs in Malaysia. Such information may also provide a basis for the development of strategies to enhance the attractiveness of specialties which have inadequate trained manpower. Similar to previous surveys, only a small proportion of the students indicated diagnostic branches, primary care, or public health as their choice for specialist training (*9,15*).

A number of studies from different countries have shown that choices of specialist training by undergraduate students vary (*1-16*). Results of these studies have shown that students are usually inclined towards medical or surgical specialties or subspecialties. The most preferred specialty of students in our study was internal medicine. Internal medicine and allied subjects were preferred by the plurality of students in our study, similar to results reported by studies in other countries (*9,10-14*). However, contrary to the findings of our study, students in Jordan and Greece preferred surgical branches over medical branches (*8,15*). Surprisingly, in a survey from Turkey, one-fourth of the students preferred subspecialty cardiology for specialization (*9*). We note that some students in our college did not indicate any specialty preference, while only a small proportion wanted to specialize in public health or primary care. This trend is similar to results reported from developed countries (*5,6,10,13,15*). Further, no one indicated a preference for a preclinical subject such as biochemistry, microbiology, or pathology; these subjects are thought to be less lucrative and are not well recognized as specialties in the medical fraternity (*8,9*).

There could be several reasons for internal medicine being medical students' most preferred specialty. Internal medicine specialists are known as 'doctor's doctors' who are always called upon for consultations by other physicians (*17*). Besides, we expect that television series like 'House' and 'ER' may also have played a role in influencing these students to choose internal medicine. Internal medicine may also have been considered as an interesting prospect because it opens up more choices for subspecialization. The students may also be aware of the growing burden of chronic non-communicable diseases in Malaysia, which increases the demand for internists (*18*). Most people want to become doctors to care for patients, and this desire matches the continuous care required by patients treated in internal medicine.

In our study, gender differences were noted in the preference for certain specialties. Female students were more likely to prefer internal medicine and allied subjects over surgical specialties. Female students were more likely to choose Ob/Gyn than their their male counterparts. This result is similar to findings from Japan, Jordan, Turkey, and Switzerland (*4,8,9,16*). Female students were more likely to choose internal medicine and allied subjects because they involve less physical work than surgical fields like general surgery and orthopedics. In Malaysia as well as the countries mentioned above, conservative Islamic societies, women generally prefer to consult a female doctor for pregnancy or gynecological problems. More women might be inclined toward Ob/Gyn for this reason. Our study showed that fourth-year and final-year students preferred medical over surgical specialties; this is in agreement with the studies reported from Jordan and the USA (*8,10,13*), where students' progress through medical school is reported to change their choice from surgical specialties to internal medicine and allied specialties.

Some studies have also explored factors that influence the choice of specialist training

(*3,4,7-9,14*). These studies have reported that financial considerations, prestige, personal competencies as well as intelligence, and clinical experience were the main factors influencing decisions to choose a specialty. We elucidated all these factors using a Likert scale. Our study found both personal and professional factors to be significant. This is in agreement with the results of studies from Japan, Canada, Jordan, Turkey, and the USA (*4,6,8-10,13,14*). Our survey found that, among the personal factors, the influence of teaching faculty and hospital consultants was the most important, while among professional factors, inspiration during clinical postings, the challenging nature of a specialty, and suitability to the student's personality were 'important'. The approach we used to determine the factors influencing the choice of subject was different from other studies from Australia (*3*), Canada (*6*), and Taiwan (*7*), which have analyzed the factors with different statistical methods, while a study from Japan used a qualitative approach (*4*). Studies from Jordan (*8*) and Turkey (*9*) only listed the factors and did not use any analytical methods. This issue of reasons or factors influencing choice of specialty is complex and often more than one factor may be involved in decision making. Future studies should focus more on a qualitative than a quantitative approach.

Most students intended to undergo specialist training in Malaysia, though some students indicated the United Kingdom and Australia as alternatives. More creditability, their better reputation, and the worldwide recognition of postgraduate courses in these countries may be the reasons for choosing these countries (*17*). We are unsure if migration was a motive for seeking entry into these countries for postgraduate training. Malaysia remained the first choice because the cost of training is nominal and the course structure is tailored to suit the Malaysian health care system and the disease burden. On the other hand, postgraduate training courses in Malaysia are available in only three public universities.

However, our survey was conducted only among clinical-phase students from one private medical school. Therefore, our findings may not reflect the true picture of medical students' choice of specialty in all medical schools in Malaysia. Though our response rate was fairly high, more female than male students responded, we cannot rule out some selection bias.

5. Conclusion

Though internal medicine and allied specialties and surgical specialties were indicated as preferred subjects for specialization, the students surveyed were not inclined towards primary care or diagnostic specialties. Incentives and other measures must be put in place to make these branches more attractive to young doctors. Qualitative studies are necessary to assess the complex issue of factors influencing choices of specialty. Further studies should be carried out on a representative sample of students at other medical schools of Malaysia.

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Original Article

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Impaired function of bone marrow-derived endothelial progenitor cells in murine liver fibrosis

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Summary **Liver fibrosis (LF) caused by chronic liver damage has been considered as an irreversible disease. As alternative therapy for liver transplantation, there are high expectations for regenerative medicine of the liver. Bone marrow (BM)- or peripheral blood-derived stem cells, including endothelial progenitor cells (EPCs), have recently been used to treat liver cirrhosis. We investigated the biology of BM-derived EPC in a mouse model of LF. C57BL/6J mice were subcutaneously injected with carbon tetrachloride (CCl_4) every 3 days for 90 days. Sacrificed 2 days after final injection, whole blood (WB) was collected for isolation of mononuclear cells (MNCs) and biochemical examination. Assessments of EPC in the peripheral blood and BM were performed by flow cytometry and EPC colony-forming assay, respectively, using purified MNCs and BM c-KIT$^+$, Sca-1$^+$, and Lin$^-$ (KSL) cells. Liver tissues underwent histological analysis with hematoxylin/eosin/Azan staining, and spleens were excised and weighed. CCl_4-treated mice exhibited histologically bridging fibrosis, pseudolobular formation, and splenomegaly, indicating successful induction of LF. The frequency of definitive EPC-colony-forming-units (CFU) as well as total EPC-CFU at the equivalent cell number of 500 BM-KSL cells decreased significantly ($p < 0.0001$) in LF mice compared with control mice; no significant changes in primitive EPC-CFU occurred in LF mice. The frequency of WB-MNCs of definitive EPC-CFU decreased significantly ($p < 0.01$) in LF mice compared with control mice. Together, these findings indicated the existence of impaired EPC function and differentiation in BM-derived EPCs in LF mice and might be related to clinical LF.**

Keywords: Liver fibrosis, carbon tetrachloride, endothelial progenitor cells, differentiation

1. Introduction

Since the discovery of endothelial progenitor cells (EPC) in 1997 (*1*), many researchers have demonstrated the critical role of bone marrow (BM)-derived EPC in postnatal vasculogenesis through pivotal bioactivities, mobilization, homing, migration, differentiation, and proliferation in angiovasculogenic tissues (*2*).

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Based on this 'dogma', the number of circulating EPC is considered to reflect *in vivo* vasculogenic activity under pathophysiological conditions. For example, in a physiological feature, the menstrual cycle of human females is reportedly to lead to cyclic fluctuations in the number of circulating EPC (*3*).

On the other hand, pathological features, such as cardiovascular risk factors, including coronary heart disease or heart failure (*4*), diabetes mellitus (*5*), hypercholesterolemia (*6*), and smoking (*7*) have been reported to decease the number of circulating EPC, indicating an impairment of EPC differentiation and/or mobilization. Recently, chronic inflammatory disorders, such as ulcerative colitis (*8*), rheumatoid arthritis (*9*),

uremia (*10*), and Alzheimer's disease (*11*) also have been reported to decrease the number of BM-derived EPC, indicating the potential value of monitoring circulating EPC levels as a diagnostic biomarker of individual diseases.

However, in hepatological view, there are few scientific or clinical studies on EPC kinetics in chronic inflammatory liver injury. Using a murine liver fibrosis (LF) model induced by injection of carbon tetrachloride (CCl_4), we recently used an EPC colony forming assay of ancestral stem/progenitors, lineage negative, c-KIT$^+$, Sca-1$^+$, and Lin$^-$ (KSL) cells to show that BM-EPC production was impaired in LF.

In the present study, we used our established model to further investigate BM-derived EPC bioactivity, particularly its potential for differentiation in LF.

2. Materials and Methods

2.1. *Animals*

Male C57BL/6J mice were purchased at 6 weeks of age from Crea Japan, Inc. (Tokyo, Japan). All animal experiments were conducted in accordance with the institutional guidelines of Tokai University School of Medicine (Isehara, Japan).

2.2. *Preparation of the liver fibrosis model*

To induce LF, mice were subcutaneously injected with CCl_4 (1 mL/kg body weight) mixed with olive oil every 3 days for 90 days. Control mice were injected with olive oil alone (*12*). All mice were sacrificed 2 days after the last injection. After liver tissues were obtained, hematoxylin and eosin (H-E) and Azan staining were performed. In addition, the animals' bodies were weighted, and their spleens also were separately weighed.

2.3. *Isolation of whole blood mononuclear cells (WB-MNCs) and biochemical examinations*

Blood samples were collected by intracardiac needle aspiration into heparinized containers. MNCs and plasma were isolated by density-gradient centrifugation (Histopaque 1083; Sigma-Aldrich, Missouri, USA), and erythrocytes were lysed with ammonium chloride. Nucleated cells were washed twice with phosphate-buffered saline/ethylenediaminetetraacetic acid (PBS-EDTA) to eliminate platelets. Biochemical examinations of blood were performed by the Central Clinical Laboratory of Tokai University Hospital (Isehara, Japan) and SRL (Atsugi, Japan). Blood was analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ-GTP), alkaline phosphatase (ALP), total bilirubin, total protein, albumin, and hyaluronic acid (HA).

2.4. *Isolation of BM-KSL cells*

A BM stem cell population of KSL cells was isolated by cell sorter, FACS Aria (Becton Dickinson; BD, New Jersey, USA), according to the reported protocol (*13*), as described below. Whole BM cells were harvested from mouse tibias and femurs as described previously(*14*), then washed with PBS-EDTA, followed by ammonium chloride hemolysis to remove erythrocytes.

Isolated BM-MNCs were initially stained for 20 min at 4ºC with a lineage-positive antibody cocktail containing CD45R/B220 (BD), TER119 (BD), CD3e (BD), CD11b (BD), Ly-6G, and Ly6C (Gr-1) (BD). After labeling the lineage positive (Lin$^+$) antibodies with biotin-labeled magnetic beads, cells were subjected to a negative selection process with a magnetic cell sorting system (MACS). Lin-negative (Lin$^-$) cells were counted and then incubated for 20 min at 4ºC with rat-fluorescein isothiocyanate anti-mouse Ly-6A/E (Sca-1) (BD) and rat-PE CD117 (c-KIT) (BD), washed three times, and resuspended in 20% Iscove's modified Dulbecco medium (GIBCO, Invitrogen, California, USA). Fluorescein isothiocyanate-conjugated Sca-1 and phycoerythrin-conjugated c-KIT double-positive cells were then obtained by FACS.

The absolute number of BM stem cells was calculated by multiplying the proportion of KSL cells by the total number of Lin$^-$ cells.

2.5. *EPC colony forming assay*

The vasculogenic potential of BM-KSL cells as a stem cell fraction of BM-derived EPCs (*15*) was assessed using an EPC colony-forming assay. A total of 500 BM stem cells per dish were seeded into a 35-mm hydrophilic tissue culture dishes (Falcon, BD), and EPC colony-forming-units (CFUs) were counted 7 days later. To characterize the EPC colony-derived cells, adherent colonies were stained with Alex 488-conjugated Griffonia simplicifolia isolectin B4 (GS-IB4) (Invitrogen) and 1,1'-Dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiI-acLDL) (Biomedical Technologies, Wisconsin, USA) as previously described (*5*). In brief, after removing the methylcellulose by gently washing with ice-cold PBS twice, 1 mL of EBM-2 (Lonza, Basel, Switzerland) medium (5% fetal bovine serum) containing 10 μL of DiI-acLDL and 2 μL of Alex-488-conjugated GS-IB4 was added and further incubated for 3-5 h. After staining the colonies with 4',-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, California, USA), cells were photographed through a fluorescence microscope (IX70; Olympus, Tokyo, Japan).

3. Results

3.1. *Assessment of LF-mice model*

In control mice, surfaces of the liver sections were smooth, and fatty changes, characterized by large droplets in hepatocytes, were observed (Figures 1A and 1B). On the other hand, in the LF group, surfaces of the liver sections were irregular, bridging fibrosis, pseudolobule formation, and micronodular formation (Figures 1C and 1D). Spleens of CCl_4 injected mice were larger than those of the control group (Table 1). The ratio of spleen weight/body weight was significantly increased in CCl_4 mice, compared with control mice ($p < 0.005$). Serum AST, ALT, and ALP were significantly increased in CCl_4 mice compared with control mice ($p < 0.005$) (Table 2). However, there were no significant difference in mice serum γ-GTP, total bilirubin, total protein, albumin, and HA levels (data not shown). Finally, LF was clearly evident in all mice injected with CCl_4.

3.2. *Impaired commitment and differentiation potentials of BM-KSL cells in LF mice*

EPC colony forming activity of BM-KSL cells was performed by EPC colony-forming assay to morphologically detect two types of EPC-CFUs, that is, primitive EPC-CFU and definitive EPC-CFU with small round and large spindle cells, respectively as previously described (*16*) (Figure 2). Both types of EPC-CFUs featured typical endothelial aspects of acLDL-DiI uptake and isolectin B4-FITC binding (Figures 2C and 2F).

In the EPC-colony forming assay of BM-KSL cells, the number of definitive EPC-CFU ($p < 0.0001$) and total EPC-CFU ($p < 0.05$) significantly decreased in LF mice, compared with controls, although primitive EPC-CFU did not differ between groups (Figure 3). These findings indicate that the differentiation potentials of BM-KSL cells into endothelial lineage are impaired in LF mice.

3.3. *Attenuated EPC differentiation potential in circulation of LF mice*

In the EPC-colony forming assay of WB-MNCs, the number of definitive EPC-CFU ($p < 0.005$) and total EPC-CFU ($p < 0.01$) significantly decreased in LF mice, compared with controls, although primitive EPC-colony forming cells did not differ between groups (Figure 4).

4. Discussion

In the current study, we successfully prepared an LF-

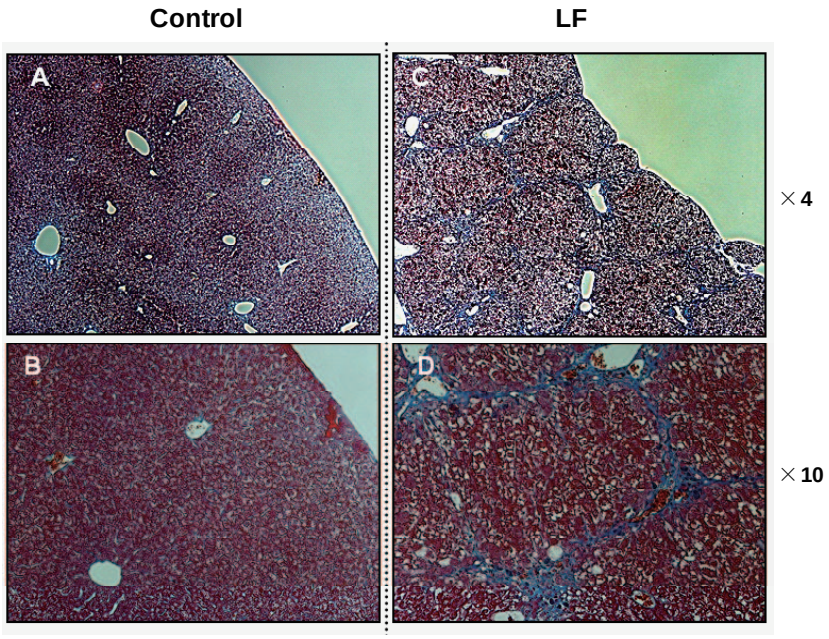

Figure 1. Liver fibrosis was observed in all mice injected with carbon tetrachloride (CCl_4). (A) and **(B)** Control mice (Azan stain: A, ×4; B, ×10). **(C)** and **(D)** CCl_4 mice (Azan stain: C, ×4; D, ×10)

Table 1. Spleen weight/body weight ratios in LF and control mice

Group	Spleen weight (mg) (Mean ± S.E.M.)	Spleen weight/Body weight (Mean ± S.E.M.)
Control	82.7 ± 3.6	2.86 ± 0.13
LF	93.5 ± 3.5	3.58 ± 0.13*

* $p < 0.005$ *vs.* control mice.

Table 2. The serum AST, ALT, and ALP levels in LF and control mice

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control	23.1 ± 2.1	20.4 ± 3.8	152.6 ± 3.5
LF	233.8 ± 2.4*	207.1 ± 38.9*	201.6 ± 5.7*

* $p < 0.005$ *vs.* control mice.

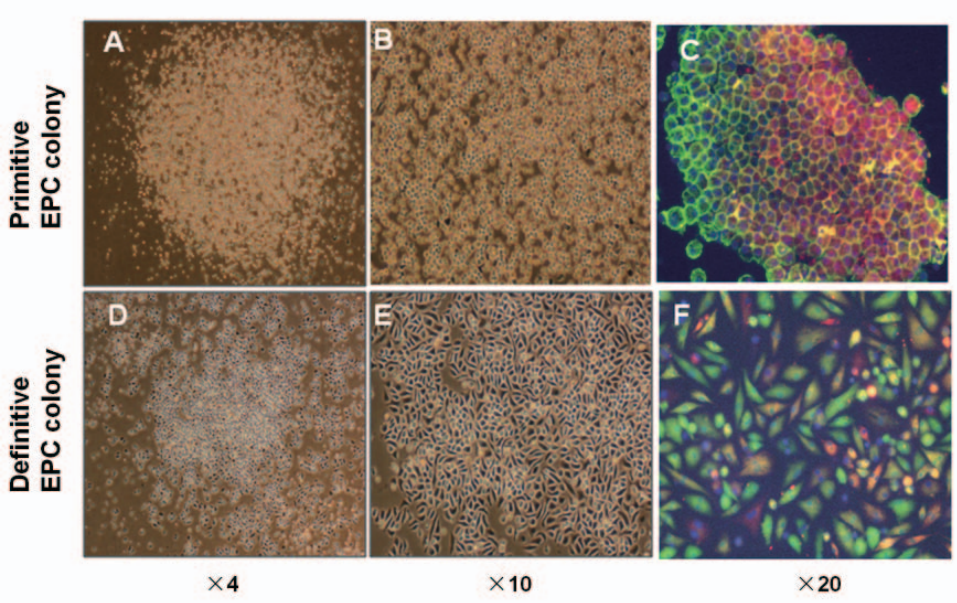

Figure 2. EPC colony-forming assay revealed two distinct colonies. (A) and **(B)** Primitive EPC colony (A, ×4; B, ×10). **(C)** and **(F)** Both colonies expressed acLDL (red), isolectin B4 (green), and DAPI (blue) cells (C and F, merge ×20). **(D)** and **(E)** Definitive EPC colony (D, ×4; E, ×10).

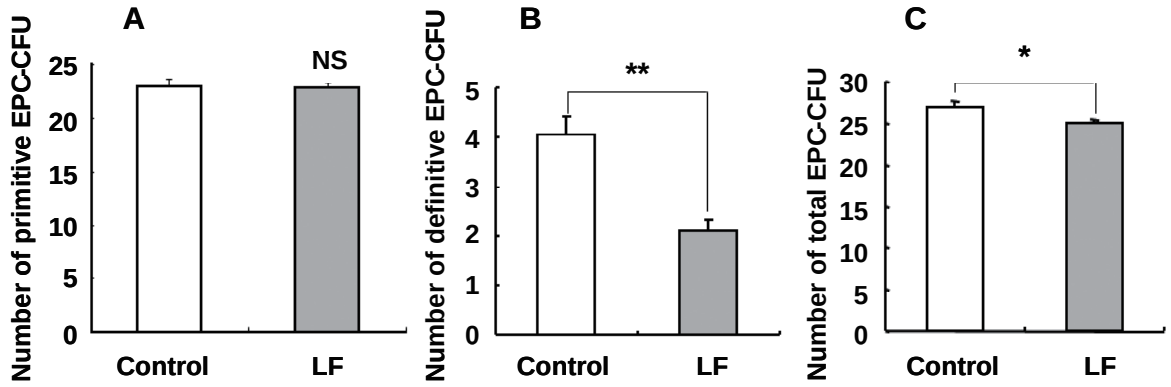

Figure 3. In BM-KSL cells, the number of definitive EPC colony-forming-units was significantly decreased in LF-mice. (A) Number of primitive EPC colony-forming-units. **(B)** Number of definitive EPC colony-forming-units. **(C)** Number of total EPC colony-forming-units. Control group ($n = 9$), LF group ($n = 13$). * $p < 0.05$, ** $p < 0.0001$ *vs.* control mice.

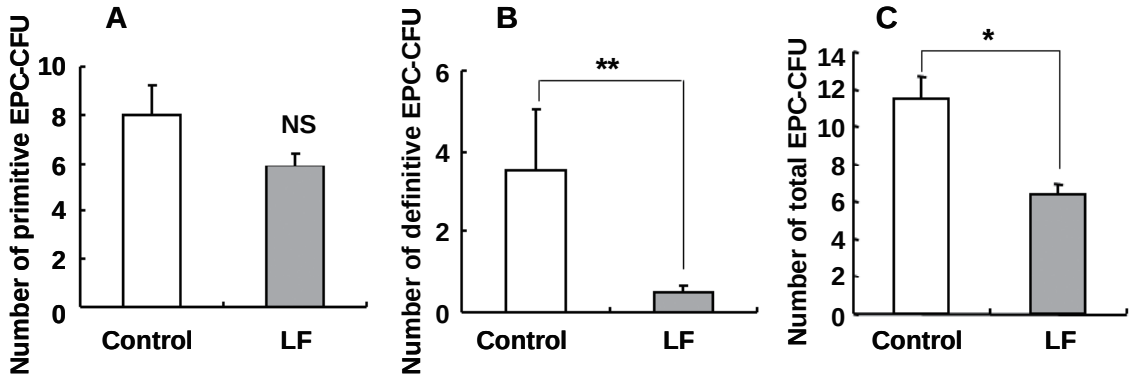

Figure 4. In WB-MNCs, the number of definitive EPC colony-forming-units was significantly decreased in LF-mice. (A) Number of primitive EPC colony-forming-units. **(B)** Number of definitive EPC colony-forming-units. **(C)** Number of total EPC colony-forming-units. Control group ($n = 4$), LF group ($n = 13$). * $p < 0.01$, ** $p < 0.005$ *vs.* control mice.

mice model *via* CCl_4 administration to evaluate EPC kinetics in LF using an EPC colony forming assay. Results showed that in BM and within the circulation of LF mice, the frequency of primitive colony forming EPC was not attenuated, but that of definitive colony forming EPC was significantly decreased, resulting in a decreased number of whole colonies forming EPC in LF-mice *versus* controls. These findings indicate that the function of EPC differentiation is downregulated in LF mice.

Chronic inflammatory LF induces vascular remodeling exerted by proangiogenic growth factors produced under hypoxic conditions *via* inadequate blood supply triggered by fibrotic tissue in humans (*16*). Based on the progression of LF, the replacement of functional hepatocytes with an excess amount of extracellular matrix is considered to decrease the production of proangiogenic growth factors or cytokines, such as vascular endothelial growth factor (*17*), cyclooxygenase-2-induced eicosanoids (*18*), hepatocyte growth factor (*19*), and thrombopoietin (*20*).

Various studies have demonstrated the pivotal roles of BM-derived EPCs, including their role in postnatal vasculogenesis, that is, mobilization, differentiation, and in foci recruitment, and shown that these cells are upregulated by proangiogenic growth factors, cytokines, and hormones (*2*). Previous reports show that EPC induce vasculogenesis and repair the injured tissues in several diseases (*21*,*22*). One of the mechanisms of this effect is that EPCs express several growth factors that exert paracrine effects for tissue regeneration, including hepatocyte growth factor, vascular endothelial growth factor, and transforming growth factor-alpha (*23*). Therefore, it is predicted that disturbed EPC differentiation might give rise to inadequate production for tissue regeneration, although the productivity of growth factors accompanying EPC differentiation need to be investigated.

Alternatively, it has recently been reported that the physiological homeostasis of EPC function is impaired in patients with diabetes (*5*), hypertension (*24*), hyperlipidemia (*6*), rheumatic disease (*9*), Alzheimer disease (*11*), aging, and smoking (*7*). In these conditions, oxidative stress is a key factor in inducing senescence or apoptosis of EPCs, resulting in impairments of physiological functions of EPC (*25*). Also, oxidative stress has been demonstrated to be a major inducer of pathological progression into LF in chronic liver disease as well (*26*). Therefore, in the present study, it is understandable that oxidative stress causing LF may impair EPC differentiation potential, as previously discussed in diabetes (*16*,*27*).

A therapeutic vascular improvement induced by using EPCs for chronic lower-limb ischemia including arteriosclerosis obliterans or Burger's disease, as well as myocardial infarction or angina pectoris, has been identified (*28*). In the field of liver regeneration therapy, autologous bone marrow cell infusion therapy, using MNC (so-called non-selected EPCs), or selected EPCs ($CD34^+$ or $CD133^+$) has been used to treat liver cirrhosis patients in clinical studies, and favorable therapeutic efficacy has been reported (*29*,*30*). However, although accumulating evidence has indicated that EPC therapy may be a useful treatment for liver cirrhosis patients, there have been few reports on EPC kinetics during the development of LF.

Our study demonstrated that the homeostasis of EPC function was disrupted in LF, indicating a weakened regenerative capability. Given these results, it appears necessary to precisely and adequately investigate the efficacy of autologous bone marrow cell infusion using 'naïve EPCs' or to develop a novel strategy for improvement of EPC differentiation potential. Regarding this, G-CSF is reported to promote VEGF secretion from neutrophil for angiogenesis (*31*). Accordingly, the administration of G-CSF may be considered as one of strategies to repair its potential by induction of EPC differentiation as well as mobilization, because VEGF augments EPC differentiation and mobilization (*32*). The transplantation of EPCs after G-CSF administration may further enhance the recovery from LF. Alternatively, *ex vivo* culture system to improve the impaired EPC function, when successfully developed, might be another promising strategy.

In conclusion, LF exhibits impaired EPC commitment and differentiation potentials, providing information applicable not only for the pathological diagnosis, but also for the development of an effective EPC therapy.

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Original Article

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Immunohistochemical characterization of the cellular infiltrate in discoid lupus erythematosus

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Summary **Discoid lupus erythematosus (DLE) is a chronic connective tissue disease of unknown etiology, but immunologic factors may play an important role in the pathogenesis. We investigated the features of immunohistochemical characterization of the cellular infiltrate in DLE. Skin samples were obtained from 5 patients using a 6 mm punch biopsy. Samples were stained with monoclonal antibodies against CD1a, CD3, CD4, CD8, CD20, CD25, CD30, and CD57. The number of cells stained with each monoclonal antibody was calculated. The number of cells stained with each monoclonal antibody in the dermis infiltration in DLE was calculated and all were higher than those in the normal control. The numbers of $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, $CD25^+$, or $CD57^+$ cells in DLE were statistically higher than those in normal skin ($p < 0.05$). The numbers of $CD1a^+$ and $CD30^+$ cells in DLE were appreciably increased but had no statistical significance compared with normal skin. In conclusion, this study revealed that T lymphocytes, B lymphocytes, and natural killer cells may play some roles in the pathogenesis of DLE.**

Keywords: CD panel antibodies, immunohistochemistry, T cells, B cells, natural killer cells

1. Introduction

In 1882 Kaposi (*1*) distinguished discoid lupus erythmatosus (DLE) from the systemic form (SLE). The term discoid is used to describe a coin-shaped, dark to erythematosus scaly lesion. Keratinous plugs, whitish depressed areas (atrophy), and telangiectases are also commonly seen. Hypo- and hyper-pigmentation are often residual findings (*2*). Discoid lesions are not exclusive for DLE; they are observed in 15-30% of SLE patients (*2,3*). Only 5-10% of adult patients with DLE develop SLE (*3*). Most adult patients with DLE are between the ages of 20 and 60 years (*4*), and the female/male ratio is 2:1 to 3:1 (*5*).

There is evidence for systemic immune disturbance in DLE patients, as judged by the findings of an increased CD4/CD8 ratio in the peripheral blood compared with healthy controls, hypergammaglobulinaemia, and the presence of autoantibodies (*6,7*).

In classic DLE lesions, epidermal changes include hyperkeratosis and variable atrophy. Dermal changes include a dense mononuclear cell infiltrate which usually consists of lymphocytes and plasma cells predominantly in the periappendageal and perivascular area. In active lesions, the infiltrate can be found approximating the dermal-epidermal junction associated with hydropic degeneration. A patchy inflammatory infiltrate also may be present in the upper dermis in an interstitial pattern and around eccrine coils. The infiltrate is often quite dense and typically extends well into the deeper reticular dermis and/or subcutis (*8,9*).

The purpose of our experiments is to investigate primarily the immunohistochemical characterization of the cellular infiltrate in patients with DLE.

2. Materials and Methods

2.1. *Patients*

Five patients with DLE were randomly chosen for this study, and the disease was confirmed by clinical and pathological examination. Three patients were

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males, and the rest were females. Mean age was 38.6 years. Three normal specimens as controls were also obtained from age- and sex-matched healthy volunteers. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki.

2.2. *Cutaneous samples*

A skin fragment of the lesion was obtained from each patient by biopsy. The specimen was fixed in 10% formalin for 24 h and processed by routine procedures for embedding in paraffin. Histological sections (4 μm) were stained with hematoxylin/eosin (HE). The remaining material was used for immunohistochemical analysis.

2.3. *Immunohistochemistry*

Serial sections were prepared from formalin-fixed, paraffin-embedded skin samples. Monoclonal antibodies (mAb) specific for CD1a (1590, Immunotech, Marseille, France), CD3 (PS1, Novocastra Laboratories, Newcastle, UK), CD4 (IF6, Novocastra Laboratories), CD8 (C8/144, Dako, Glostrup, Denmark), CD20 (NJ1, Novocastra Laboratories), CD25 (4C9, Novocastra Laboratories), CD30 (182, Novocastra Laboratories), CD57 (NK1, Novocastra Laboratories) were used for primary staining. Secondary staining was performed using the LSAB2 staining kit (Dako) for 30 min at room temperature.

Serial 4 μm thick sections were mounted on silane slides (Dako) and submitted to fixation, deparaffinization in xylene and rehydration through graded alcohols. The antigen was retrieved in 0.1 M Tris-HCl buffer (pH 9.5, containing 5% urea) using a microwave oven for 15 min, each for antigen recovery of the molecules. After cooling to room temperature for 50 min, the slides were treated with 0.3% hydrogen peroxide in methanol (Merck, Darmstadt, Germany) for 30 min to block endogenous peroxidases. Nonspecific staining was blocked with 5% normal horse serum for 30 min. Subsequently the slides were incubated with the primary antibodies diluted in horse serum albumin at 4°C overnight in a humidified chamber. The samples were treated with biotinylated secondary antibody (horse anti-mouse) for 30 min. After washing with PBS, the slides were incubated with ABC reagent for 60 min at room temperature. The slides were then stained with DAB solution for 2-4 min under a microscope at room temperature. Finally the reaction was terminated by washing in distilled water. The slides were washed with PBS between each reaction step. Samples in which the primary antibody was omitted were used as a negative control. Positive labeling was identified by a brown staining around the cell membrane or by a brown color of the cytoplasm according to the marker employed.

All sections were examined in an Olympus BX50 light microscope (Tokyo, Japan) and results were expressed as the mean count of cells per 10 high-power fields (400×). Ten microscopic fields, representing the densest cellular inflammatory infiltrate, were selected per specimen and positive cell numbers were estimated as a proportion of lesion area, using a point counting method.

The scale corresponded to the percentage of stained cells with specific antibody each time, compared to the total cellular infiltration and counting was scored as following: –, no cells (negative); +, few cells (0~10%, weak); ++, some cells (10~25%, moderate); +++, many cells (25~50%, intense); ++++, plenty of cells (more than 50%, very intense).

2.4. *Statistical analysis*

All the data were collected, classified and entered into a spreadsheet for statistical analysis, using the *t*-test. The dermal infiltration of DLE and normal skin were compared for the following cell counts (per HPF): CD1a, CD3, CD4, CD8, CD4/CD8, CD20, CD25, CD30, and CD57. Results are given as mean ± S.D. $p < 0.05$ was considered significant.

3. Results

3.1. *Evaluation of CD4/CD8 ratio*

The CD4/CD8 cell ratio is known to be an indicator of the host immunoregulatory status. The ratio of CD4/CD8 ranged from 0.7 to 1.7 in the five DLE patients. The mean was 1.0 (Table 1), and there was no statistical significance compared to that in normal skin (1.1).

3.2. *The percentage of each positive cell in the infiltrates*

Examining serial sections, light microscopy revealed that the inflammatory response was concentrated perivascular and periappendageal. The infiltrates consisted mostly of lymphocytes and plasma cells. The percentages of positive cells for each CD antibody are listed in Table 2.

The CD1a mAb stained all five lesions. Most of them (4/5) showed weak infiltration with a mean percentage of 6.7%. Besides the infiltrate in the dermis, there were also many $CD1a^+$ cells in the epidermis (not calculated). Representative pictures are shown in Figures 1A and 1B.

The CD3 mAb stained all five sections to a very intense degree. The mean percentage was 69.0%. The pictures are given in Figures 1C and 1D.

The CD4 and CD8 mAb stained all five lesions to an intense degree and the mean percentage of CD4 was

Table 1. CD4/CD8 ratio in the lesions examined

Patient No.	1	2	3	4	5	Mean	Control	*p* value
CD4/CD8 ratio*	1.7	0.8	0.9	0.7	0.9	1.0	1.1	> 0.05

* The number of $CD4^+$ cells divided by the number of $CD8^+$ cells.

Table 2. The percentage of each positive cells in the infiltrates

Cell counts	CD1a	CD3	CD4	CD8	CD20	CD25	CD30	CD57
–	0	0	0	0	0	0	1	0
+	4	0	0	0	2	4	4	2
++	1	0	0	0	3	1	0	3
+++	0	0	5	5	0	0	0	0
++++	0	5	0	0	0	0	0	0
Mean (%)	6.7	69.0	34.5	37.8	11.6	7.5	3.5	11.3

–, negative; +, weak (0~10%); ++, moderate (10~25%); +++, intense (25~50%); ++++, very intense (> 50%).

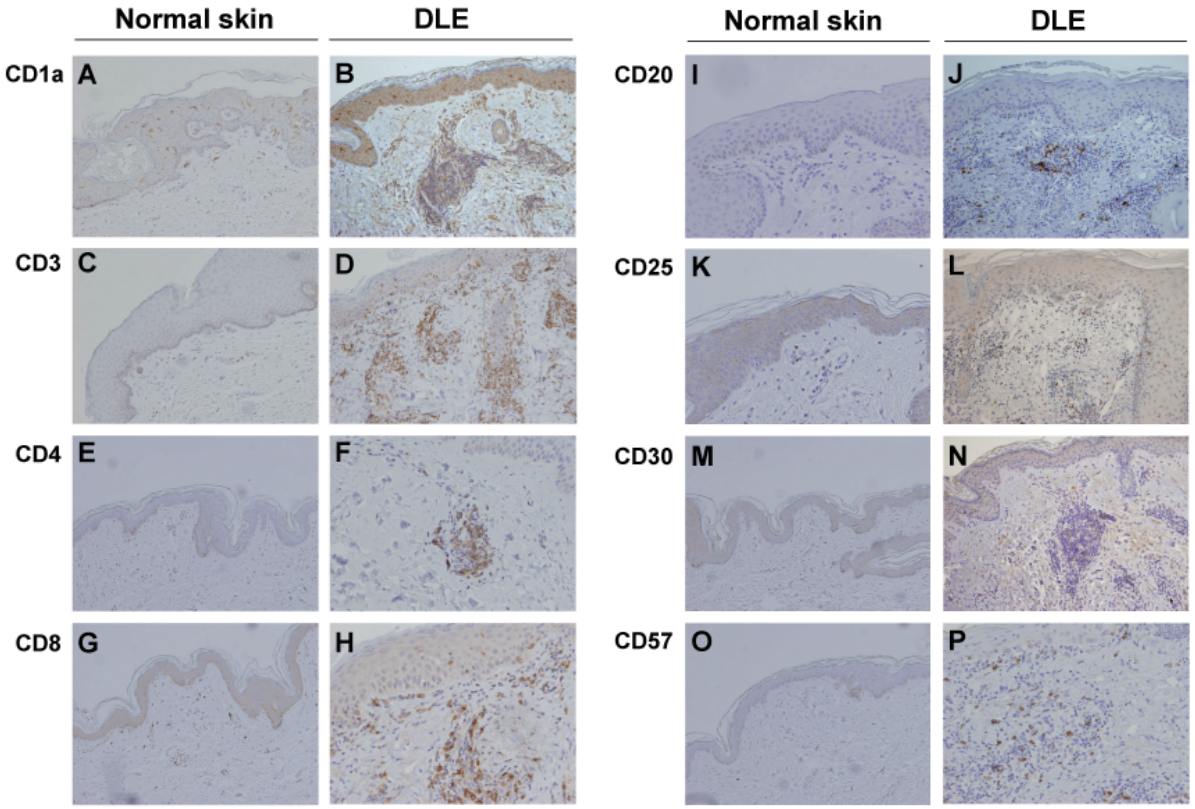

Figure 1. Immunohistochemical staining of CD panel antibodies. The anti-CD1a mAb stained a few infiltrate cells around many lymphocytes in DLE (**A**, normal skin; **B**, DLE). The anti-CD3 mAb stained many infiltrate cells in the dermis (**C**, normal skin; **D**, DLE). The anti-CD4 mAb stained many infiltrate cells around vessels (**E**, normal skin; **F**, DLE). The anti-CD8 mAb stained many infiltrate cells around vessels (**G**, normal skin; **H**, DLE). The anti-CD20 mAb stained some infiltrate cells among the lymphocytes (**I**, normal skin; **J**, DLE). The anti-CD25 mAb stained a few infiltrate cells around vessels (**K**, normal skin; **L**, DLE). The anti-CD30 mAb stained only a few infiltrate cells around many lymphocytes (**M**, normal skin; **N**, DLE). The anti-CD57 mAb stained some infiltrate cells among many lymphocytes (**O**, normal skin; **P**, DLE). Original magnification, ×100.

34.5%, while that of CD8 was 37.8%. The expressions of the two mAbs are exhibited in Figures 1E, 1F, 1G, and 1H.

CD20 positive cells were observed in all five skin lesions. Two of them were weak, the other three were moderate. The mean percentage was 11.6%. The pictures are shown in Figures 1I and 1J.

$CD25^+$ cell infiltrates were weak in four sections and moderate in one with a mean percentage of 9.4%. Representative pictures are shown in Figures 1K and 1L.

The CD30 mAb stained four lesions to a weak degree, while the other was negative. The mean

percentage was less than 3.5%. The expression of CD30 is exhibited in Figures 1M and 1N.

$CD57^+$ cell infiltrates were weak in two and moderate in three with a mean percentage of 11.3%. The pictures are shown in Figures 1O and 1P.

3.3. *Positive cell numbers of immunostaining with anti-CD1a, anti-CD3, anti-CD4, anti-CD8, anti-CD20, anti-CD25, anti-CD30, and anti-CD57 mAbs*

All lesional skins showed dermal perivascular and periappendageal infiltration. The results of numbers for each positive cell in per HPF are summarized in Table 3. The numbers of $CD3^+$ (94.1 ± 51.8), $CD4^+$ (48.3 ± 18.2), $CD8^+$ (55.9 ± 27.9), $CD20^+$ (21.3 ± 16.4), $CD25^+$ (6.7 ± 1.9), and $CD57^+$ (11.8 ± 4.8) cells in DLE were significantly higher than those in normal skin ($p < 0.05$). Although the numbers of CD1a (2.3 ± 1.3) and $CD30^+$ (4.0 ± 3.3) cells in DLE were also higher than those in normal skin, there was no statistical difference between them.

4. Discussion

DLE is a connective tissue disorder characterized by well-demarcated, erythematous, slightly infiltrated "discoid" plaques that often show adherent thick scales and follicular plugging. The etiology of DLE is still unknown but considered to be multifactorial at this time. Immunologic factors may play an important role in its pathogenesis. In active lesions, the dense inflammatory infiltrate is observed in the dermal-epidermal junction. The infiltrate in dermis is often quite dense in the periappendageal and perivascular area and typically extends well into the deeper reticular dermis and subcutis.

The present immunohistochemical study was conducted to show the features of a panel of CD monoclonal antibodies in dermal infiltrate in the skin of DLE patients.

Analysis of the lymphocyte population by immunohistochemistry revealed a conspicuous infiltrate of T lymphocytes in the dermis of DLE patients which were usually observed around appendices and blood vessels. CD3 positive cells were found in the dermal infiltrate of all biopsies and showed a considerable percentage in the infiltrating area (69.0%). The number of $CD3^+$ T cells per HPF (94.1 ± 51.8) in DLE was significantly higher than that in normal skin (5.8 ± 5.3, $p < 0.05$). Amoura *et al*. (*10*) suggested that chronic discoid lupus erythematosus (CDLE), the most common clinical subtype of cutaneous lupus erythematosus (CLE), was characterized by a dense lymphocytic infiltrate composed of $CD3^+$ lymphocytes with a slight predominance of $CD4^+$ over $CD8^+$.

T lymphocytes are critical as mediators of inflammation immunity through the combined activity of $CD8^+$ and $CD4^+$ cells. Immunohistochemical analysis of different subsets of T cells revealed the host immunoregulatory status. There are many more $CD4^+$ T cells (48.3 ± 18.2) and $CD8^+$ T cells (55.9 ± 27.9) in the dermal infiltration than those of normal controls (2.9 ± 1.0 and 2.5 ± 0.6, respectively). It was detected that the number of $CD8^+$ cells were appreciably higher than $CD4^+$ cells in the DLE lesions. This was appreciably different from other authors (*11*,*12*), but a similar result was also obtained by Wenzel *et al*. (*13*). The CD4/CD8 ratio, which ranged approximately from 0.7 to 1.7 (mean 1.0), was a slight decrease compared to that in normal skin (1.1), but there was no statistical significance between them. Wouters *et al*. (*14*) studied the circulating lymphocyte profiles in patients with DLE and found that the levels of $CD4^+$ T cells were similar in DLE patients and healthy controls, but that $CD8^+$ T cells were decreased both in absolute numbers and percentages, which resulted in a mild increase of the CD4/CD8 ratio. The decreased number of circulating $CD8^+$ T cells found in their study might result from a shift of these cells to the skin that may be involved in T-cell mediated cytotoxic damage of basal keratinocytes. This may be compatible with the immunohistological finding of $CD8^+$ T cells close to damaged keratinocytes in lesional epidermis (*14*).

In this study we determined the presence of B lymphocytes in cutaneous lesions based on the CD20 marker. CD20 is a protein solely expressed on B

Table 3. Results of immunohistochemical staining

CD mAb	Patients ($n = 5$)	Control ($n = 3$)	p value
$CD1a^+$ (per HPF dermis)	2.3 ± 1.3	1.1 ± 0.1	> 0.05
$CD3^+$ (per HPF dermis)	94.1 ± 51.8	5.8 ± 5.3	< 0.05
$CD4^+$ (per HPF dermis)	48.3 ± 18.2	2.9 ± 1.0	< 0.05
$CD8^+$ (per HPF dermis)	55.9 ± 27.9	2.5 ± 0.6	< 0.05
CD4/CD8 ratio	1.0 ± 0.4	1.1 ± 0.2	> 0.05
$CD20^+$ (per HPF dermis)	21.3 ± 16.4	0.6 ± 0.9	< 0.05
$CD25^+$ (per HPF dermis)	6.7 ± 1.9	2.5 ± 1.8	< 0.05
$CD30^+$ (per HPF dermis)	4.0 ± 3.3	0.5 ± 0.7	> 0.05
$CD57^+$ (per HPF dermis)	11.8 ± 4.8	2.4 ± 0.6	< 0.05

Data are mean ± S.D. $p < 0.05$ was considered statistical significance.

lymphocytes (*15*). In our sections, B cells were found in perivascular aggregates, surrounded by T lymphocytes. The number of $CD20^+$ cells (21.3 ± 16.4) in the infiltration of DLE lesions was significantly higher than that in normal skin (0.6 ± 0.9) and there was a statistical significance ($p < 0.05$). The mean percentage of $CD20^+$ cells was 11.6% of the inflammatory cells. Previous studies reported that B lymphocytes were rare or absent (< 5%) in DLE lesions (*11*,*16-18*). However, Akasu *et al*. observed B lymphocytes accounted for > 25% in one of his five DLE cases (*19*). Wolfgang *et al*. found B lymphocytes were over 5% of the inflammatory cells in 31% of their DLE cases and in 8 of 49 cases, and that B lymphocytes accounted for > 20% of the inflammatory cell infiltrate. In short, our study substantiated Akasu and Wolfgang's findings that numerous B lymphocytes may be found in DLE lesions. In a circulating lymphocyte study (*14*), the percentage of $CD19^+$ B cells was increased in DLE patients compared with healthy controls, which was compatible with earlier studies that demonstrated increased percentages of cytoplasmic immunoglobulin-containing and immunoglobulin-secreting cells in the DLE peripheral blood. The role of B lymphocytes in DLE is unknown, but local secretion of specific antibodies is possible, which may act through mechanisms such as opsonization and activation of the complement system.

CD25 is the marker for human interleukin (IL)-2 receptor. In this study the percentage of $CD25^+$ cells in dermal inflammatory cells was relatively low (6.7 ± 1.9), just as previously described by other authors (*11*,*20*,*21*), however, it was slightly increased compared to that in normal skin (2.5 ± 1.8) and there was statistical significance. Following the activation of T cells with antigen or mitogen in the presence of the IL-1, IL-2 is rapidly synthesized and secreted. In response to this, a subpopulation of T cells expresses high affinity receptors for IL-2. These cells proliferate and expand the T cell population which is capable of mediating helper, suppressor and cytotoxic functions. The activation of T lymphocytes by IL-2 suggests that non-specific, non-major histocompatibility complex (MHC)-restricted mechanisms of cellular cytotoxicity is involved in the pathogenesis of the skin lesions in LE (*11*).

CD30 or Ki-1 antigen expression was rarely seen in dermal DLE infiltration. Comparably, normal skin controls in this study showed less positive findings. CD30 has been suggested to be a marker for Th2 cells (*22*). Our result indicates that the Th2 cells may not be included in the dermal immunoreaction in DLE.

We found an increased number (11.8 ± 4.8) and percentage (11.3%) of $CD57^+$ NK cells in DLE compared with normal skin and there was statistical significance. NK cells are thought to play an important role in tissue damage as well as in modulation of B cell activity (*23*). Wouters *et al*. (*14*) found a lower number of $CD57^+$ cells in peripheral blood in DLE. They hypothesized that the reduction of these subsets in peripheral blood may be the result of skin recruitment. Our result provided evidence for their hypothesis. Their study proved the systemic activation of the cellular immune system in DLE.

The result of this study revealed that T lymphocytes, B lymphocytes cells, and natural killer cells may play some roles in DLE pathogenesis. The inflammatory effector mechanisms of DLE may be confined to the skin and mediated by T cells, B cells, and NK cells which may migrate from the circulation to the site of inflammation. The number of patients included in this study was not large. Further studies are needed to analyze the *in situ* participation of key cytokines on these cells to better understand the pathogenesis of this disease.

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