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Keratin 17 identified by proteomic analysis may be involved in tumor angiogenesis

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Angiogenesis is crucial for solid tumor growth. By secreting angiogenic factors, tumor cells induce angiogenesis. However, targeting these angiogenic factors for cancer therapy is not always successful, suggesting that other factors may be involved in tumor angiogenesis. This work shows that 25 protein spots were differentially expressed by two-dimensional gel electrophoretic analysis when HepG2 cells induced endothelial cell differentiation to tube *in vitro*, and most of them were upregulated. Twenty-one proteins were identified with MALDI-TOF-MS, and the other four were identified by LTQ-MS/MS. Keratins were identified as one class of these upregulated proteins. Further study indicated that the expression of keratin 17 in cultured endothelial cells is likely microenvironment regulated, because its expression can be induced by HepG2 cells and bFGF as well as serum in culture media. Increased expression of keratins in endothelial cells, such as keratin 17, may contribute to the angiogenesis induced by HepG2 cells. [BMB reports 2009; 42(6): 344-349]

INTRODUCTION

Angiogenesis, the formation of new vascular capillaries and networks, is a crucial component of various biological processes including embryonic development and tumor growth (1). Solid tumor growth is highly angiogenesis dependent. Without angiogenesis, tumor nodules cannot grow beyond 2 to 3 mm in diameter because of the lack of sufficient blood supply (2). To grow further, a tumor must form its own capillary network invading the tumor mass by stimulating angiogenesis in response to direct or indirect interactions between tumor cells and endothelial cells. It is known that when cells in tumor tissues are deprived of oxygen, some angiogenic factors are released. bFGF (basic fibroblast growth factor) is one of the most commonly expressed angiogenic factors (3, 4), which can act as both a mitogen and a chemoattractant for endothelial cells during tumor angiogenesis (5). It is reported that bFGF is present in the tumor tissue at a higher concentration than in the normal tissue in which angiogenesis takes place (6). Such angiogenic factors are important targets for anticancer therapy. But targeting these factors in human cancer therapy has not always been effective, suggesting that other factors or components may also play a key role in tumor angiogenesis (7-9). Identification of these factors may have important implication in cancer therapy.

Tumor angiogenesis is a complex phenomenon that involves multiple steps, including invasion of endothelial cells into a stroma space, cell migration and proliferation, and later differentiation to form new blood vessels (10). The collagen gel assay (11) has been developed to model specific steps of angiogenesis (migration, proliferation and tube formation) *in vitro*. In this model system, endothelial cells can be induced to form tube-like structures when cocultured with tumor cells (7, 12). It is a suitable model for the analysis of early regulation of angiogenesis stimulated by tumor cells. Differential proteomic analysis is a potent strategy for identifying those factors and components implicated in tumor angiogenesis at the protein level. New proteins that are related to angiogenesis could be identified by using the coculture model and undertaking a comparative proteomic analysis, which may lead to a better understanding of the key regulatory events in angiogenesis *in vitro*.

RESULTS

Identification of proteins differentially expressed in cocultures and individual cultures

For tube formation assay, HUVECs were mixed and cultured together with HepG2 cells on collagen type I as cocultures. Individually cultured cells were used as the control. Our studies showed that the number of tube-like structures in cocultures was more than that in individual cultures at 18 h, suggesting that HepG2 cells could positively influence the formation of tube-like structures when endothelial cells were cocultured with tumor cells (Fig. 1). To identify key effectors in this event, we utilized a proteomic approach with 2-DE and MS. 2-DE

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maps of cocultures and individual cultures obtained between pH 3 and 11 displayed approximately 1,700 spots each. Twenty-five spots that were differentially expressed in cocultures, as compared with individual cultures, were then chosen for further MS analysis. The proteins present in 21 spots were identified with MALDI-TOF-MS, and the proteins of the other four spots were identified by LTQ-MS/MS. The corresponding profiles and 25 differentially expressed proteins are shown in

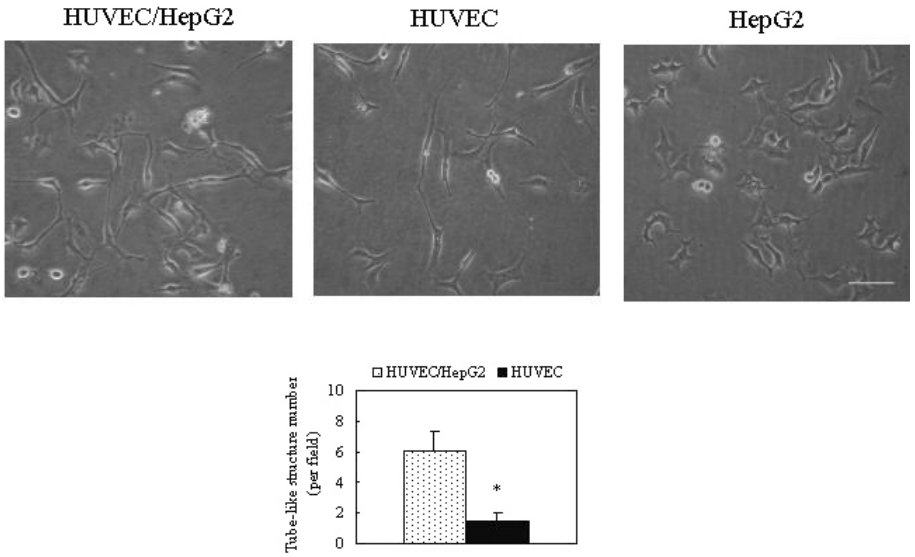

Fig. 1. Promotion of endothelial tube-like structure formation by HepG2 cells. For coculture experiments, HUVECs and HepG2 cells were plated on collagen gel, and HUVECs or HepG2 cells on collagen gel for controls, respectively. After 18 h, tube-like structures were counted in ten microscopic fields (200×) in each of five replicates and averaged (mean ± SD from five independent experiments). *P < 0.01 vs coculture (HUVEC/HepG2). Bar, 50 μm.

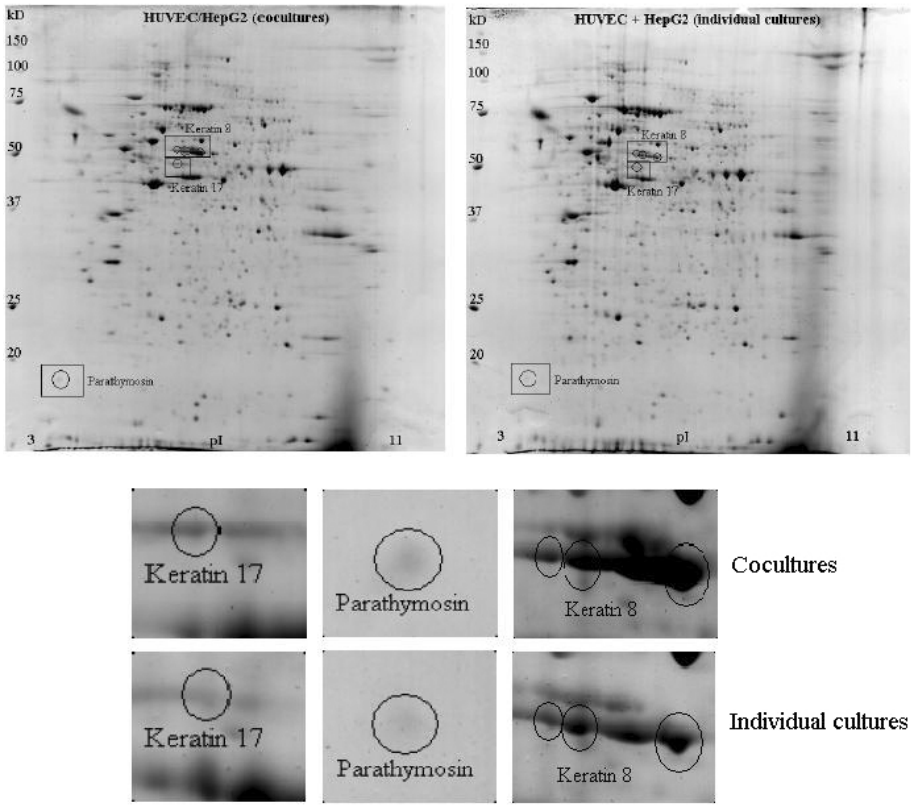

Fig. 2. Differentially expressed proteins in cocultures and individual cultures. 2D electrophoresis patterns of whole-cell proteins isolated from cells in cocultures cells or in individual cultures on collagen gel were shown. Three differentially expressed proteins are indicated.

Fig. 2 and supplemental Table S1, respectively. Twenty-four identified proteins were upregulated in the cocultured cells as compared with the individually cultured cells. Based on the information obtained from the Swiss-Prot and NCBInr websites, ten of the identified proteins are involved in the regulation of the cytoskeleton, reflecting the dramatic cytoskeletal reorganization required to form new vascular tubes. Others are related to protein synthesis and signaling transduction, etc.

Validation of differentially expressed proteins

To validate the proteomics results, RT-PCR and Western blot analysis for some identified proteins were performed. Fig. 3 showed a representative RT-PCR result of K17, K8 and Parathymosin mRNA expression in cocultures and individual cultures, as well as cultures of one type of cells (i.e. HepG2 cells and HUVECs). Fig. 3 also showed the Western blot results for K17. These results showed that the expression levels of K17, K8 and Parathymosin were higher in cocultures compared with individual cultures, which is consistent with the proteomics results. These results also indicated that HUVECs and HepG2 cells both expressed K17 (Fig. 3).

K17 expression in HUVECs stimulated by HepG2 cells

As shown in Fig. 4A, K17 expressions were obviously upregulated in HUVECs when cocultured with HepG2 cells in Millicell units, which may explain, at least partially, a significant enhancement of the K17 expression in cocultures comparing with controls in the tube formation assay. Western blot analysis also showed that keratin 17 protein was differentially expressed in HUVECs maintained in DMEM medium with 10% or 2% FBS (Fig. 4B), which suggests that K17 expression in HUVECs is likely associated with the microenvironment, and its expression may be induced by serum containing some growth factors. When HUVEC cells were cultured in DMEM with 2% serum and human bFGF, we found that K17 expression in HUVECs was significantly increased (Fig. 4B).

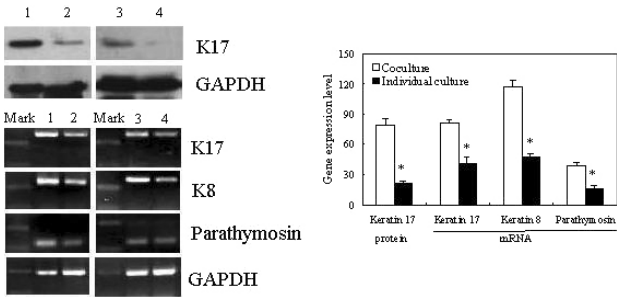

Fig. 3. Validation of changes in K17, K8 and Parathymosin by RT-PCR and Western blot analysis (1: coculture; 2: individual culture; 3: HepG2; 4: HUVEC). Western blot results for K17 were shown (Mean ± SD from four independent experiments). RT-PCR results for K17, K8 and Parathymosin were shown (Mean ± SD from five independent experiments). *P < 0.01 vs coculture.

DISCUSSION

The eukaryotic cytoskeleton is composed of three distinct elements, the microtubules, the microfilaments and the intermediate filaments (IFs). Among them, IF proteins have been used to identify the origin of specific cells. For example, keratins were found in epithelial cells and vimentin were found in mesenchyme-derived cells (fibroblasts and endothelial cells) (13). So far, over 70 different IF proteins, belonging to six major categories, have been identified. Although their exact functions remain unclear, IFs likely play a variety of nonstructural roles, including cell motility, cell-cell and cell-extracellular matrix interactions, receptor-ligand interactions, and receptor internalization (14).

In the present study, we used a proteomic approach to identify the differentially expressed proteins in the cocultures of HUVECs and HepG2 cells. Ten of these proteins are related with the functions of cytoskeletons, and keratins account for most of them. HepG2 is a human hepatocellular carcinoma cell line. Because it is an epitheliallike cell, it can express epithelial keratin proteins. Although hepatocyte IFs are made solely of keratins 8 and 18, the keratin expression profile of hepatocytes may change with malignant transformation. For example, HepG2 also expressed keratin 19 (15). K17 expression in HepG2 cells were also found in this study. Although keratin expression in mesenchymal cells such as endothelial cells remains controversial, many reports reveal that keratins may be expressed by certain endothelial cells, such as

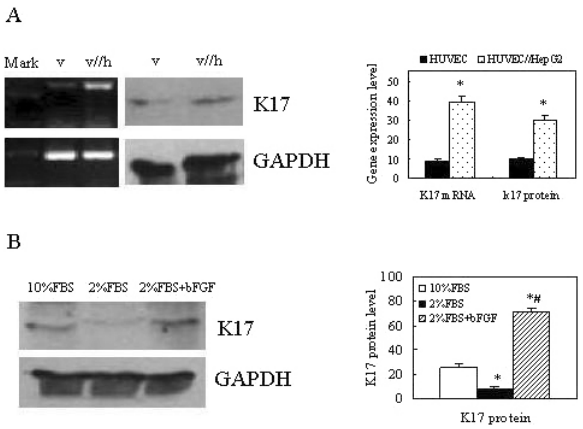

Fig. 4. K17 expression in HUVECs. (A) HUVECs were cultured for 24 h in DMEM media with 10% FBS, 2% FBS and 2% FBS + 10 ng/ml bFGF, respectively. Western blot results showed that bFGF increased K17 expression in HUVECs (Mean ± SD from four independent experiments). *P < 0.01 vs 10% FBS, #P < 0.01 vs 2% FBS. (B) HUVECs (v) were cocultured with HepG2 (h) cells in Millicell units in DMEM media with 2% FBS (v//h: v cocultured with h). Western blot and RT-PCR results showed increased K17 expression in HUVECs induced by HepG2 cells (Mean ± SD from four independent experiments). *P < 0.01 vs HUVEC.

cultured endothelial cells and endothelial cells of blood vessels in synovial and submucosal tissues (16-19). These data are consistent with our RT-PCR and Western blot findings showing that keratin expression is present in cultured endothelial cells.

Angiogenesis involves several primary steps such as proliferation, migration and differentiation. Cell migration is regulated by a network of intrinsic factors and external stimuli, involving changes in the cytoskeleton, cell-substrate adhesions and the extracellular matrix (20). Studies by Katagata showed that keratins in cultured fibroblasts and endothelial cells, including K17, only existed as the extremely thin fiber-like substances but not as the filamentous structures (17). This finding suggests that keratin IFs in cultured fibroblasts and endothelial cells may act as signal transducers rather than mechanical components. Moreover, many studies suggest that IFs relay signals from the extracellular matrix to the intracellular compartment likely via the transmembrane integrins (21). Integrin-mediated signaling may have a crucial effect on cell migration (22, 23). In our model, we demonstrated that HepG2-promoted formation of tube-like structures were accompanied by a significant increase in the expression of keratins. Coculture of HUVECs with HepG2 cells in Millicell units led to enhanced K17 expression in HUVECs. Based on these results, we speculate that elevated keratins in HUVECs, which could augment HUVEC migration, might partially explain the increased tube-like structures in cocultures.

The induction of tumor angiogenesis is mediated by the release of angiogenic factors (24). Among these factors, bFGF is a wide-spectrum angiogenic factor shown to induce migration of endothelial cells both *in vivo* and *in vitro* (5). Our results showed that human bFGF, as well as HepG2, induced the increased expression of K17 in HUVECs. It is possible that HepG2 cells increased keratin expression in HUVECs by secreting bFGF. Collectively, promotion of tube formation by HepG2 cells may be associated with the increased keratin expression of HUVECs stimulated by angiogenic factors. And, the elevated keratin expression likely increases endothelial cell motility, which remains to be determined.

It is of interest to investigate the keratin expression of endothelial cells in tumor vasculature and circulating endothelial progenitor cells implicated in tumor angiogenesis, which may provide a novel target for the vascular targeting therapy of tumors. Such studies are currently under consideration.

MATERIALS AND METHODS

Tube formation assay

Human umbilical vein endothelial cells (HUVEC) and HepG2 cells were maintained in DMEM medium with 10% FBS (Hyclone, USA). Tube formation experiments were performed referring to the method of Bohman *et al*. (25). For coculture assay, the cells were washed with serum-free DMEM, then 1×10^5 HUVEC and 1×10^5 HepG2 cells were mixed and kept in medium containing 2% serum in 25 cm^2 flasks coated with type I collagen (Sigma-Aldrich Co., USA). For controls (individual culture), 1×10^5 HUVEC and 1×10^5 HepG2 cells were cultured, respectively. The flasks were then incubated at 37°C for 18 h. The cells were examined as described below, and the tube structures (25) were counted.

Protein sample preparation and two-dimensional gel electrophoresis

For each culture condition, two types of samples were collected, namely individually cultured cells for control samples (HUVEC + HepG2) and cocultured cells for treated samples (HUVEC/HepG2). After incubation in the conditioned medium for 18 h as above, cells were harvested, and whole cell protein extracts were prepared for two-dimensional gel electrophoresis (2-DE) analysis using lysis buffer (8M urea, 4% CHAPS, 20 mM Tris, 60 mM DTT, 1 mM PMSF). Protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, California, USA). 2-DE was performed as described previously (26). Briefly, 500 micrograms of total proteins were mixed with rehydration buffer (8M urea, 2% CHAPS, 50 mM DTT, 0.5% IPG buffer) and loaded on IPG dry strips ($180 \times 3 \times 0.5$ mm, pH3-11 nonlinear, GE Healthcare, Sweden), then isoelectrically focused on an IPGphor™ IEF system (Amersham Biosciences, Sweden). The second dimension was run with the Ettan DALTsix vertical gel electrophoresis system (Amersham Biosciences, Sweden) on 12% polyacrylamide gels. The proteins were detected after Coomassie Blue G250 (Bio-Rad Laboratories, California, USA) staining. The above processes were made in triplicate for each sample.

Image analysis

The Coomassie blue-stained images were recorded using an Image Scanner (Amersham Biosciences, Sweden) at a resolution of 300 dots per inch (dpi). Intensities of protein spots were analyzed with ImageMaster 2D Platinum Software v5.0 (Amersham Biosciences, Sweden). To exclude variations due to protein loading and staining, the spot volume was normalized and the expression level was determined by the relative volume of each spot to the total volume over all the spots present in the gel. To test the influence of coculture versus individual culture conditions, groupwise statistical comparisons were performed on groups of spots from triplicate gels both in coculture and individual culture. Variations of the expression level were calculated as the ratio of average values of the relative volume for a group between the two types of samples. Only protein spots with a variation ratio greater than 3.0 were considered differentially expressed. Statistical significance of differences between groups was determined by the Student's t test ($P < 0.05$).

In-gel digestion of protein, mass spectrometry (MS)

Candidate protein spots were cut off from the gels and extracted by in gel digestion as described by Wang *et al.* (27). The generated peptides were analyzed by MALDI-TOF-MS us-

ing a Bruker Daltonics AutoFlex TOF-TOF LIFT mass spectrometer (Germany). Protein identification was carried out using the MASCOT search engine (http://www.matrixscience.com; Matrix Science, London, UK), and the NCBI nonredundant protein database was used for peptide search. Unidentified peptide mixtures by MALDI-TOF-MS were measured using a LTQ Orbitrap system (Thermo Finnigan, San Jose, CA, USA). Peptides and proteins were identified using Bioworkers Software (Thermo Finnigan), which uses the acquired MS/MS spectra to search against the IPI human v3.26 protein database. The protein identification criteria were based on Delta CN ($\geq$ 0.1) and SEQUEST cross-correlation value (one charge $\geq$ 1.9, two charges $\geq$ 2.2, three charges $\geq$ 3.75).

RT-PCR analyses

Extracts of RNA from cultured cells were prepared by using Trizol reagents (Invitrogen, USA). After synthesis of the first strand cDNA, the following pairs of primers were used for PCR: keratin 17 (323bp fragment), 5′-TTCCGCACCAAGTTTGA GACA-3′ (forward) and 5′-AAGAACCAATCCTCGGCATCC-3′ (reverse); keratin 8 (318bp fragment), 5′-TTCCGCACCAAGTT TGAGACA-3′ (forward) and 5′-AAGAACCAATCCTCGGCATC C-3′ (reverse); Parathymosin (139bp fragment), 5′-AGTTGAGC GCCAAGGACCT-3′ (forward) and 5′-TCTCCATCCTCGGCAG TTTC-3′ (reverse), and GAPDH (240bp fragment), 5′-TGATGA CATCAAGAAGGTGGTGAAG-3′ (forward) and 5′-TCCTTGGA GGCCATGTGGGCCAT-3′ (reverse). The ratio of analyzed RT-PCR products to the internal standard (GAPDH) was used to represent gene expression. Primers spanning an intron and reverse transcription reactions without reverse transcriptase were utilized to exclude genomic DNA contamination.

Western blot analyses

Equal amounts of protein extracts from cultured cells were resolved by SDS-PAGE on 12% polyacrylamide gels, and transferred onto PVDF membrane (Boehringer Mannheim GmbH, Germany) with an semi-dry transfer apparatus (Hoefer TE70, Amersham Biosciences, Sweden). The membrane blots were incubated with 1/1,000 vol/vol diluted mouse anti-Cytokeratin peptide 17 monoclonal antibodies (Clone CK-E3, Sigma-Aldrich Co., USA), and then probed with 1/50,000 vol/vol diluted horseradish peroxidase-conjugated anti-mouse IgG and revealed with an ECL reagent from Pierce (Rockford, IL, USA). The Western blot results were analyzed with the Quantity One 1-D analysis software (Bio-Rad Laboratories, California, USA).

HepG2 or bFGF-induced K17 expression of HUVEC cells

Coculturing procedure involving 6-well Millicell units with 0.4 μm PCF filters (Millipore, USA) was used to determine whether HepG2 cells can induce keratin 17 (K17) expression in HUVEC cells. HUVEC cells (3×10^5) were seeded on the apical side of the membrane and HepG2 cells (6×10^5) on the plastic feeder plate. HUVEC cells (3×10^5) on the apical side of the membrane without HepG2 cells on the plastic feeder plate were used as control. After incubation in DMEM with 2% serum for 24 h, HUVEC cells were harvested for RT-PCR and Western blot analysis of K17 expression.

HUVEC cells were cultured for 24 h in DMEM medium supplemented with 10% FBS, 2% FBS and 2% FBS + 10 ng/ml human bFGF (preparation of human bFGF as described previously (28)) respectively, and then were harvested for Western blot analysis of K17 expression.

Statistical analysis

Using SPSS14.0, statistical difference between groups was determined by ANOVA. All results are presented as mean $\pm$ SD. A P value less than 0.05 was considered statistically significant.

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