Journal of Cellular Biochemistry 102:463–472 (2007)

Evaluation of Bone-Derived And Marrow-Derived Vascular Endothelial Cells by Microarray Analysis

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Abstract This study focused on the differential expression levels of proteins that may exist between bone-derived and marrow-derived vascular endothelial cells (BVEC and MVEC). The vascular cells were isolated from trabecular bone regions and central marrow cavity regions of mouse long bones. Cells were cultured for 1 week to expand the population then separated from non-vascular cells using biotinylated isolectin B4, streptavidin-coated metallic microbeads, and a magnetic column. After an additional week of culture time, RNA was isolated from both cell types and compared using microarray analysis. RT-PCR was used to confirm and relatively quantitate the RNA messages. The bone-derived cells expressed more aldehyde dehydrogenase 3A1 (ALDH3A1), *S*ecreted *M*odular *C*alcium-2 (SMOC-2), CCAAT enhancer binding protein (C/EBP-β), matrix metalloproteinase 13 (MMP-13), and annexin 8 (ANX8) than the marrow-derived cells. Spα and matrix GLA-protein (MGP) were produced in greater abundance by the marrow-derived cells. This study reveals that there are profound and unique differences between the vasculature of the metaphysis as compared to that of the central marrow cavity. The unique array of proteins expressed by the bone-derived endothelial cells may support growth of tumors from cancer cells that frequently metastasize and lodge in the trabecular bone regions. J. Cell. Biochem. 102: 463–472, 2007. © 2007 Wiley-Liss, Inc.

Key words: vascular endothelial cells; bone vasculature; microarray

The metaphyses of long bones, vertebrae, ribs, and the pelvic girdle are highly vascularized and contain a meshwork of trabecular bone. These sites are preferred sites for metastastic breast and prostate cancer cells to lodge and develop into tumor masses. Metastasis of cancer cells is a complex process involving many factors that favor the survival of cancer cells. The steps of metastasis include migration of cancer cells from the primary site into blood vessels, evasion of innate immune surveillance, adherence to vascular endothelia of distant organs, and migration out of blood vessels and into the secondary tissue sites [Welch et al., 2000]. Cancer cells that have arrived in secondary tissue sites first encounter vascular endothelial cells which line the blood vessels. The structure of the vascular bed in metaphyseal bone contributes to the entrapment of the cancer cells. Instead of being formed into a network of small diameter capillaries found in most tissues, the vasculature of metaphyseal bone consists of voluminous sinusoids with a lumenal diameter which is several times the diameter of the cancer cells. Blood flow in the large sinusoids is slow compared to capillary beds in other tissues. A reduced rate of flow would allow time for the slowly moving cancer cells to adhere to the vascular lining cells. Furthermore, vascular lining cells in regions of trabecular bone display more E-selectin, a cell surface adhesion molecule, than in comparable cells derived from the central marrow cavity [Makuch et al., 2006].

In the present study, we continued the quest to identify differences in bone-derived vascular endothelial cells (BVEC) where breast and prostate cancer cells lodge as compared with marrow-derived vascular endothelial cells

Grant sponsor: U.S. Army Medical Research and Materiel Command; Grant number: DAMD 17-03-1-0583; Grant sponsor: National Institutes of Health; Grant number: DE 09459.

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Received 15 November 2006; Accepted 24 January 2007

DOI 10.1002/jcb.21307

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(MVEC), a site where cancer cells rarely develop into tumors. We compared the two cell types using microarray analysis and confirmed the data using RT-PCR analysis. Message for five proteins (aldehyde dehydrogenase 3A1 (ALDH3A1), *S*ecreted *M*odular *C*alcium-2 (SMOC-2), matrix metalloproteinase 13 (MMP-13), CCAAT enhancer binding protein (C/EBP-β), and annexin 8 (ANX8)) were consistently present in greater abundance in the bone derived cells, whereas two proteins (Spα and matrix GLA-protein (MGP)) were more abundant in the marrow derived cells. These findings are discussed in terms of possible roles for each protein.

MATERIALS AND METHODS

Isolation of Bone- and Marrow-Derived Endothelial Cells

Eleven female Swiss Webster mice, 7–9 weeks of age were euthanized by CO_2 inhalation. Tibias and femurs were removed and stripped of extraneous muscle and connective tissue. The bones were split longitudinally, exposing the central marrow cavity and metaphysis. Marrow was removed from the central third of the bone shaft and placed in DMEM (Sigma-Aldrich, St. Louis, MO); remaining marrow in the shaft was removed and discarded. Loose marrow and blood cells were flushed from the metaphyses and discarded. Metaphyses were then scraped with a No.1 curette (1 mm diameter) into DMEM to obtain trabecular bone fragments and sinusoidal endothelial cells.

BVEC and MVEC were obtained from the crude metaphyseal and marrow isolates, respectively, by treatment with 0.1% Type 1A collagenase (C-9891, Sigma-Aldrich) for 30 min at 37°C. These preparations were centrifuged at 1,200 rpm and resuspended in Medium 199 (MediaTech, Herndon, VA) supplemented with 20% fetal bovine serum (Sigma-Aldrich), 1% penicillin/streptomycin (MediaTech) and 1× endothelial cell growth factor (ECGF) from Sigma-Aldrich. Cells were plated in 60 mm tissue culture dishes coated with 2% gelatin and incubated at 37°C with 5% CO_2 humidified atmosphere. Adherent cells were cultured for 7 days in order to increase the cell number for subsequent study; media was changed every other day.

Endothelial cells were separated from other cell types in the crude isolate cultures using the Miltenyi VarioMACS (Auburn, CA) magnetic cell sorting system. After 1 week of culture, cells were released with a cell dispersion solution, Accutase (Innovative Cell Technologies, San Diego, CA) and resuspended at a density not exceeding 5×10^6 cells/500 μl in a labeling buffer consisting of PBS with 2 mM EDTA. The isolectin B4 from *Griffonia* (*Bandeirea*) *simplicifolia* seeds binds preferentially to mouse endothelial cells [Marelli-Berg et al., 2000] and so was used to label the endothelial cells for magnetic sorting. The biotinylated isolectin B4 (Vector Technologies, Burlingame, CA) was diluted 1:50 in each cell suspension and incubated at 4°C for 30 min. Cells were rinsed twice with labeling buffer then resuspended in 90 μl of labeling buffer. Magnetic microbeads coated with streptavidin (Miltenyi Biotec) were added to labeled cell suspension and incubated at 4°C for 15 min. Cells and beads were washed twice with labeling buffer and resuspended in 500 μl of separation buffer consisting of PBS with 2 mM EDTA and 0.5% calf serum (Sigma-Aldrich). One MACS–MS separation column (Miltenyi Biotec) for each cell type was prewashed with separation buffer and placed in the VarioMACS magnetic stand. Cell suspensions were then loaded into the reservoir of each column and allowed to flow through the columns. Cells that were not labeled with the biotinylated isolectin lacked streptavidin-coated magnetic microbeads attached to their surfaces and were not retained in the column. All columns were rinsed twice with separation buffer then removed from the magnetic stand. Magnetically labeled endothelial cells retained in the column were then flushed out in 1 ml of separation buffer. Cells suspensions were spun at 1,200 rpm and resuspended in Medium 199 supplemented with 20% FBS, 1% penicillin/streptomycin and 1× ECGF. Cells were plated at a density of approximately 50,000 cells/cm^2 in gelatin coated 35 mm plates and incubated at 37°C for 7 days with media changes every other day.

Cultures of putative endothelial cells isolated by this method were checked for purity by assessing their ability to take up fluorescent labeled acetylated LDL at an accelerated rate [Voyta et al., 1984] and immunohistochemical staining for the surface marker, PECAM, as previously described [Makuch et al., 2006]. Virtually all of the cells observed from the cell preparations were positive for acetylated LDL uptake indicating a highly purified population of vascular endothelial cells.

RNA Isolation and Microarray Analysis

After 1 week in culture, BVECs and MVECs were harvested. Total RNA was extracted from each cell type using the Qiagen RNeasy kit (Qiagen, Valencia, CA). Microarray labeling, hybridization, and data collection were conducted by the Penn State University DNA Microarray Facility under the direction of Dr. Craig Praul. Microarray analysis was run on BVEC and MVEC RNA from three separate cell isolations. Briefly, RNA from three separate isolations of BVECs and MVECs was labeled using the Affymetrix GeneChip Expression 3′ Amplification One-Cycle Target Labeling kit (Affymetrix, Santa Clara, CA). After biotin labeling of the antisense strand, generated cRNAs were fragmented and hybridized to the Affymetrix GeneChip Mouse Genome 430A 2.0 array. Quality of the starting total RNA, the intact cRNA and the fragmented cRNA were assessed with the Agilent Bioanalyzer using the RNA 6000 Nano Lab Chip (Agilent, Paloalto, CA). Two of the RNA isolates were determined to be of superior quality for analysis. Arrays were read by the Affymetrix GeneChip scanner and interpreted by GeneChip Operating software version 1.3.

Microarray data statistical analysis was performed by Qing Zhang at the Penn State University Bioinformatics Consulting Center using the Loess method to normalize data with the Limma package in RBioconductor. Two sample *t*-tests were run for each gene and the Bayes method was employed to shrink the standard error to a common value across all genes. Fold increase or decrease values were determined by comparing mRNA expression levels of BVECs to the expression levels of MVECs averaged from the two best isolations.

RT-PCR Analysis

Relative quantitative PCR was performed on RNA isolated from BVECs and MVECs to verify the different levels of expression observed on the microarrays. Total RNA was isolated from the cells using the RNeasy Mini kit (Qiagen) with on-column DNase treatment. One microgram of total RNA was reverse transcribed from random decamer primers using the RETROscript kit (Ambion, Austin, TX). One-twentieth of this reaction was then used in relative quantitative PCR reactions to determine the levels of message for each of the proteins of interest. Levels of amplified PCR product were normalized to 18S RNA using QuantumRNA 18S internal standard primers (Ambion). PCR reactions were optimized by varying the cycle number to determine the linear range of the amplification. RNA that had not been reverse transcribed was used as a negative control. PCR reactions for each of the proteins of interest were performed using the forward and reverse primers and cycle numbers listed in Table I. Thermocycler parameters for all reactions were: 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. PCR products were separated by electrophoresis on a 1.5% agarose gel in 1× Tris-borate EDTA buffer and stained with ethidium bromide. Gel documentation was performed by the Kodak Gel Logic 100 Imaging System

TABLE I. Primer Pairs, Optimum Cycle Number and Amplicon Size for Relative Quantitative RT-PCR of ALDH31A, SMOC-2, MMP-13, C/EBP-β, ANX-8, Spα, and MGP mRNA in Bone- and Marrow-Derived Vascular Endothelial Cells

Gene	Primer sequence	Cycle #	Amplicon size (bp)	Citations
ALDH31A	5′ acctgcgcaagaatgaatgg 3′ 5′ ggacacccccttttgatcact 3′	24	360	[a]
SMOC-2	5′ gctcacgttcttgagagtcg 3′ 5′ tgtagctgtgacactggacc 3′	31	304	Vannahme et al. [2003]
MMP-13	5′ gatgacctgtctgaggaag 3′ 5′ atcagaccagaccttgaag 3′	23	357	Leclerc et al. [2004]
C/EBP-β	5′ggtttcgggacttgatgcaatc 3′ 5′ caacaaccccgcaggaacat 3′	22	130	Yagi et al. [2004]
ANX-8	5′ acacagatgccttggctctg 3′ 5′ gtctgcaagcttcagcatgg 3′	32	360	Stein et al. [2005]
Spα	5′ tgggagaacaactgtacccatggc 3′ 5′ aggctgagggaaaggtgtctaaag 3′	26	468	Kuwata et al. [2003]
MGP	5′ tgcgctggccgtggcaaccct 3′ 5′ cctctctgttgatctcgtaggca 3′	28	181	Bostrom et al. [2001]

[a]Primers designed from Genbank sequence NM_007436 with Primer3 software developed by the Whitehead Institute for Biomedical Research.

(Eastman Kodak, Rochester, NY) and band volume quantitation was done by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Microarray analysis revealed that mRNA encoding five proteins were expressed in greater abundance by the vascular endothelial cells derived from the trabecular bone regions (BVECs), as compared with MVECs and two proteins were lower in abundance (Table II). Proteins in greater abundance in BVECs were ALDH3A1, SMOC-2, MMP-13, C/EBP-β, and ANX8. The two proteins in lower abundance were SPα and MGP.

RT-PCR was performed to corroborate the microarray data and to relatively quantify levels of mRNA in the two types of cells. In all cases, the RT-PCR results verified the microarray findings. The message for ALDH3A1 was 4.4–12.3-fold greater in BVECs than in MVECs (Fig. 1A) while SMOC-2 message was 2.1–31-fold greater (Fig. 1B). The RT-PCR for C/EBP-β showed one result of slightly greater expression in MVECs than in BVECs; the two other trials showed 8.1–9-fold higher expression in BVECs (Fig. 1C). MMP-13 and ANX8 were expressed 2.1–31.5-fold (Fig. 1D) and 2.2–114-fold (Fig. 1E), respectively, higher in BVECs. Message for Spα was found in abundance in MVECs, but was not detectable at all in BVECs (Fig. 2A). For MGP, one isolation of cells did not reveal any difference in message between the two cell types whereas the other two cell isolations showed a 2.8- and 11.9-fold increase in the MVEC message (Fig. 2B).

TABLE II. Results of Affymetrix GeneChip Mouse Genome Array 430A 2.0 Analysis of Bone- and Marrow-Derived Vascular Endothelial Cells

Affymetrix #	Gene name	BVEC fold change over MVEC
1418752_at	ALDH3A1	+2.8
1415935_at	SMOC-2	+2.6
1417256_at	C/EBP-β	+2.1
1417256_at	MMP-13	+2.0
1425789_s_at	ANX8	+2.0
1449193_at	CD5-like antigen (Spα)	−4.2
1448416_at	MGP	−2.3

Negative fold changes denote higher expression levels in MVECs than in BVECs.

In the discussion that follows, each protein is evaluated in terms of its function in other tissues. Using this information, a perspective on the functions of these proteins in BVECs and MVECs is developed.

DISCUSSION

BVEC Proteins

When comparing microarray data, five proteins were in greater abundance in BVECs than MVECs. These data were confirmed by RT-PCR detection of message. The wide range of relative quantitative RT-PCR values may be due to variation in expression at particular phases of cell development as the cell population was increased through cell culture.

ALDH3A1. ALDH3A1 is constitutively expressed in cornea, stomach, lung, and skin [Lindahl, 1992; Vasiliou et al., 1995] and in at least some blood vessels, including aortic endothelial cells [Tampier et al., 1993] and bovine brain microvessels [Petersen, 1985]. The ALDHs form a superfamily of $NAD(P)^+$-dependent enzymes with similar primary structure and many ALDH genes have been identified across a wide spectrum of species. In humans, at least 16 ALDH genes have been identified. ALDHs are found in abundance in tissues exposed to oxidative stress. Their role is to detoxify a large variety of aliphatic and aromatic aldehydes derived from metabolism of alcohols, amino acids, biogenic amines, vitamins, steroids, and lipids. In hepatic tissue, specific ALDH genes are induced and serve to detoxify many oxidative substances encountered by the liver [Vasiliou and Pappa, 2000]. The role of ALDH3A1 and ALDH3A2 is to metabolize medium-chain and aromatic aldehydes. Many current studies focus on the role of ALDH in corneal tissue where the enzyme has been shown to promote survival of cells exposed to oxidative stress resulting from ultra-violet radiation [Choudhary et al., 2005; Pappa et al., 2005]. We report here that ALDH3A1 is present in the microvessels associated with trabecular bone at a higher level than within the marrow cavity by microarray analysis and by RT-PCR. It is possible that ALDH expression is induced in greater amounts in the trabecular bone region in response to increased levels of cytotoxic aldehydes as a consequence of production of reactive oxygen species produced by bone turnover. Active osteoclasts are known to

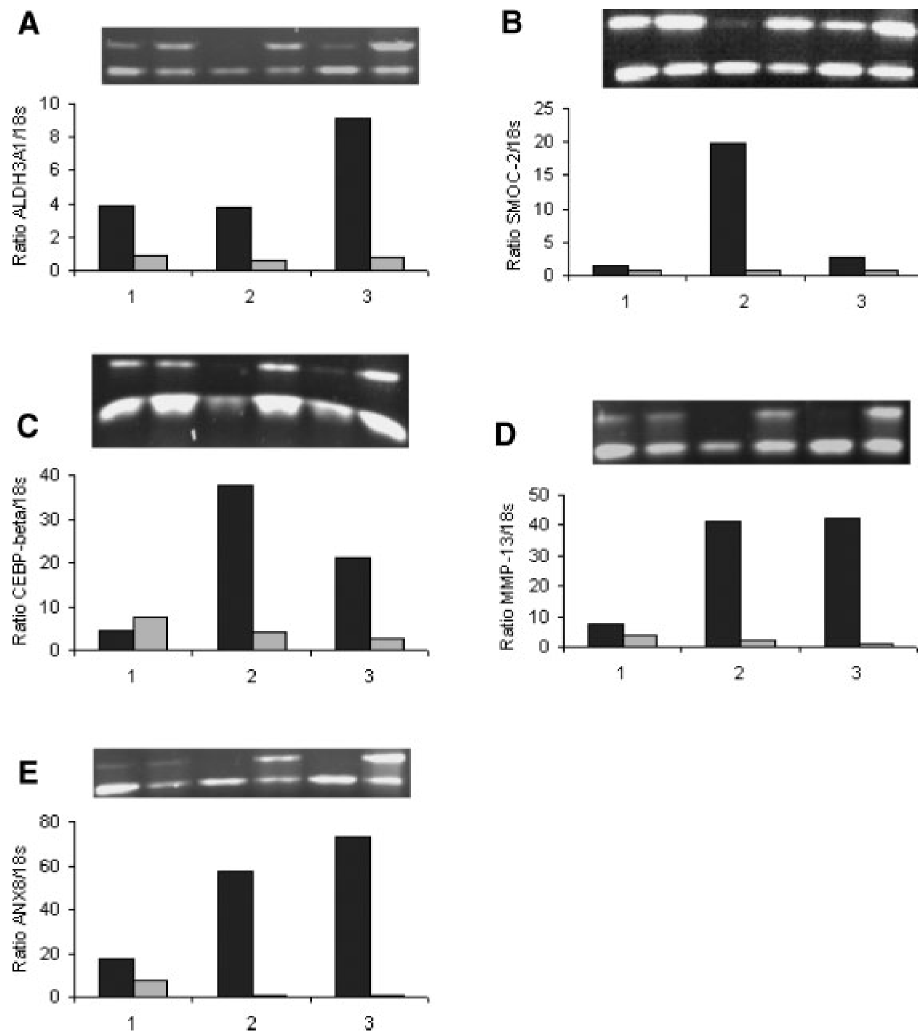

Fig. 1. Relative quantitative RT-PCR for mRNAs expressed in greater abundance in BVECs. Ethidium bromide staining of agarose gel electrophoresis and graphic representation for three isolations of BVECs and MVECs are depicted for (**A**) ALDH3A1; (**B**) SMOC-2; (**C**) C/EBP-β; (**D**) MMP-13; and (**E**) ANX8. The top band in all gel electrophoresis photographs is the 18s amplicon. In the bar graphs, BVECs are represented by (■) and MVECs are represented by (□).

produce reactive oxygen species [Vääräniemi et al., 2004]. Build-up of cytotoxic aldehydes is likely enhanced by the slow rate of blood flow in the trabecular bone regions where bone turnover is often high [Von Andrian et al., 1992; Mazo and von Andrian, 1999].

SMOC-2. The gene for SMOC-2-binding protein has been sequenced and full-length recombinant protein produced using a human embryonic kidney cell line [Vannahme et al., 2003]. SMOC-2 is a member of the SPARC family of matricellular proteins [Nishimoto et al., 2002]. SMOC-2 mRNA has been detected in a wide variety of tissues in the mouse and is particularly high in ovary, heart, skeletal muscle, and spleen. The present report and that of Rocnik et al. [2006] are the first reports of SMOC-2 in the vasculature. The human homologue of SMOC-2 has been reported to be SMAP2; it was discovered in smooth muscle during neointima formation [Nishimoto et al., 2002; Vannahme et al., 2003]. Vannahme et al. [2003] have speculated that the extracellular domain, which contains five acidic residues, has the ability to bind calcium. It is likely that Ca^{++} binding is important for the three-dimensional structure of SMOC-2. Very recently, Rocnik et al. [2006], in studies of SMOC-2 expression in human umbilical vein endothelial cells (HUVECs), found that SMOC-2 was involved

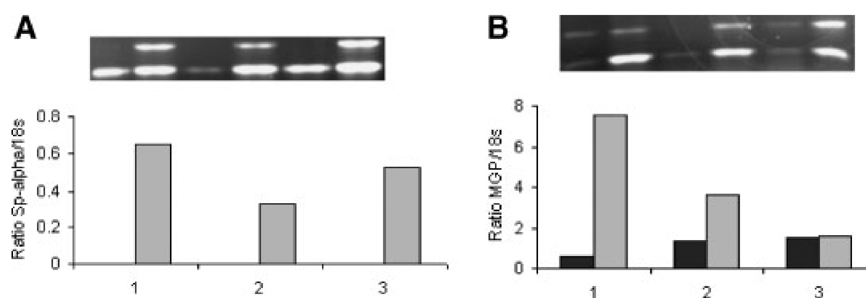

Fig. 2. Relative quantitative RT-PCR for mRNAs expressed in greater abundance in MVECs. Ethidium bromide staining of agarose gel electrophoresis and graphic representation for three isolations of BVECs and MVECs are depicted for (**A**) Spα and (**B**) MGP. The 18s amplicon is seen as the bottom band in the Spα gel electrophoresis photograph and the top band in the MGP gel. In the bar graphs, BVECs are represented by (■) and MVECs are represented by (▒).

in cell cycle progression, cell motility and microvessel tube formation. A higher level of expression and secretion of SMOC-2 in the trabecular bone region could enhance angiogenesis, not only of the trabecular bone region, but also of any tumors that develop in that region.

C/EBP-β. C/EBP-β, is found in a number of tissues during the differentiation phase. A number of C/EBP isoforms have been identified. Synthesis of C/EBP-α, C/EBP-β, and C/EBP-δ increases with development in adipose tissue of fetal and post-natal pigs [Lee et al., 1998]. Both C/EBP-α and C/EBP-β are present in preovulatory follicles; C/EBP-β is in antral folicles and early stage corpora lutea [Gillio-Meina et al., 2005]. A role for C/EBP-β has been reported in the proliferation and differentiation of osteoblasts [Iyer et al., 2004]. C/EBP-β supports proliferation and development of mammary epithelial cells [Robinson et al., 1998; Seagroves et al., 1998].

C/EBP-β, also termed NFIL-6, and has been shown to bind to a 14 base pair site in the IL-6 promoter and drive the expression of IL-6 [Yan et al., 1997]. Regulation of IL-6 expression by C/EBP-β has been shown to occur in the vasculature of lung, kidney, heart [Yan et al., 1997], and umbilical vein endothelial cells [Yan et al., 1995]. Hypoxia stimulates the C/EBP-β-driven production of IL-6, a pro-inflammatory cytokine which may exert protective effects on oxygen deprived endothelial cells. This is interesting because blood flow in metaphyseal bone, the isolation sites of the BVECs, is known to be sluggish and therefore, low oxygen tension is a property of the metaphysis [Schnitzer et al., 1982]. In addition, IL-6 acts on endothelial cells to upregulate expression of the surface adhesion molecules ICAM, VCAM, and E-selectin [Watson et al., 1996]. Breast cancer cells express the ligand for E-selectin [Renkonen et al., 1997] favoring adhesion to the endothelium of the metaphyseal vasculature. Makuch et al. [2006] have reported that osteoblasts stimulate BVECs to express more surface E-selectin.

MMP-13. It is unlikely that any MMP has a unique, irreplaceable role. However, only a few MMPs are capable of hydrolyzing the triple helical structure of collagen. These include MMP-1 (collagenase I), MMP-8 (collagenase-2), MMP-13 (collagenase-3), as reviewed by Jeffrey, 1998. MMP-13 has been shown to mediate murine and bovine aortic endothelial cell migration in a wound-healing model [Lopez-Rivera et al., 2005]. MMP-13 expression is increased substantially in vascular endothelial cells under conditions that stimulate vascular development and MMP-13 is regarded as the critical component in remodeling cellular microenvironments [Schmidt-Hansen et al., 2004]. MMPs are not constitutively expressed in most tissues; expression is stimulated by a variety of factors including bFGF, VEGF, and nitric oxide. These factors are found in the vascular environment in metaphyseal bone. The type of MMP expression stimulated by nitric oxide depends on tissue type. For vascular endothelial cells nitric oxide stimulates MMP-13 expression [Zaragoza et al., 2002]. MMPs on the surface of vascular endothelial cells have been reported to facilitate cell trafficking through the endothelial layer [Planus et al., 1999; Rajavashisth et al., 1999; Zaragoza et al., 2002; Krikun et al., 2005]. Metaphyseal bone is a region of high turnover; metaphyseal vessels are traversed by cells destined to become osteoclasts, cells which

resorb bone and initiate bone remodeling. Higher expression of MMPs in metaphyseal vessels by BVECs, as compared with MVECs, may indicate a higher turnover rate of blood vessels within the metaphysis, a phenomenon discussed by Davis and Senger [2005]. Furthermore, MMP-13 may enhance extravasation of cancer cells from the vasculature into the interstitial space that surrounds the trabecular bone meshwork [Schmidt-Hansen et al., 2004].

Annexin 8. The annexins are a multigene family of Ca^{++} binding proteins known to bind to phospholipids of membranes in a calcium-dependent manner, providing a link between Ca^{++} signaling and a variety of membrane-related events [Gerke et al., 2005; Markoff and Gerke, 2005]. The annexins are widely expressed in nature, being found among vertebrates, invertebrates, fungi, plants, and protists. The annexins have been implicated in a wide variety of physiological activities such as trafficking of membranes and membrane proteins that occurs in exocytosis, endocytosis and apoptosis, regulation of ion channels, and organization of membrane structure. ANX8, also termed VAC-β, has been reported in lung endothelia and umbilical vein endothelia, arterial smooth muscle, lung fibroblasts, and HeLa cells [Reutelingsperger et al., 1994], in growth plate chondrocytes [White et al., 2002] and in placenta [Hauptmann et al., 1989]. Interestingly, the vascular systems of many tissues do not express ANX8, for example, by the heart or spleen. Thus, ANX8 may play a unique role in some tissues. The vascular endothelium presents a passive non-thrombogenic surface and also actively interferes with coagulation. A multiplicity of anti-coagulant factors on vascular endothelia have been identified one of which, for some tissues, it is ANX8. In the present study ANX8 was expressed in greater abundance by the BVECs. This is noteworthy because slow blood flow, in the metaphyseal (or trabecular bone region) could foster coagulation; the presence of ANX8 would block coagulation in this region.

MVEC Proteins

Spα. This protein is also called CD5L or AIM (apoptosis inhibitory factor). It is a member of the superfamily of proteins defined by the presence of scavenger receptor cysteine-rich (SRCR) domains. As discussed by Gebe et al. [2000], Spα usually contains eight cysteines and thereby is in group B of the SRCR family. It has been found in invertebrates as well as in mice and humans and is highly conserved [Sarrias et al., 2004]. Spα is expressed in thymus, spleen, liver, lymph nodes, and macrophages, suggesting that it may function in development and/or maintenance of lymphoid tissue. It is unique among the SRCR proteins in that it is a soluble protein [Gebe et al., 2000]. Because macrophages secrete Spα and promote their own survival, Spα has been identified as a factor in the development of artherosclerosis [Arai et al., 2005]. It is interesting that Spα has been found to bind to a variety of bacteria [Sarrias et al., 2005]. A role for Spα in vascular endothelial cells has not been identified. Based on reports on other tissues, the role could be to promote cell development and/or survival.

Matrix Gla-protein (MGP). MGP belongs to the vitamin K-dependent, Gla-containing protein family. It is well recognized that MGP is expressed at high levels in bone, heart, kidney, lung, and the intima, and media of aorta [Shanahan et al., 1998]. More recently, several studies have reported expression of MGP in vascular endothelial cells of bovine aorta [Bostrom et al., 2004], from human myometrium treated with VEGF [Weston et al., 2002], from human dermis [Glienke et al., 2000], from human umbilical vein [Engelse et al., 2001] and from marine teleosts [Ortiz-Delgado et al., 2006]. MGP deficiency has resulted in calcification of arteries, but, interestingly, vessels which lack a thick, muscular wall comprised of intima, media, and adventicia (i.e., arterioles, capillaries, and veins) did not calcify [Luo et al., 1997]. Work by Bostrom et al. [2004] indicates that MGP may be involved in stimulating endothelial cell proliferation and migration, processes which utilized the activin-like kinase (ALK)/Smad 1 pathway. The ALK/Smad 5 pathway appeared to be involved at a later stage of vascularization. In our study we found MGP in both BVECs and MVECs, with the MVECs showing greater expression. The role of MGP in BVECs and MVECs and why MVECs express higher levels of MGP is not yet understood. One possibility is that in MVECs MGP supports constant renewal of the endothelial cells that form the sinusoids through which extensive lymphoid cell trafficking occurs. In BVECs, which are in close proximity to regions of constant bone turnover, MGP may serve to protect the sinusoidal endothelial cells and their

underlying basement membrane from being calcified.

In conclusion, this study examined the differential expression of genes in vascular endothelial cells isolated from two distinct regions of the long bone, namely the central marrow cavity and the metaphysis. Each region constitutes a unique environment, exposing the endothelial cells of that region to a different array of transcription factors, cytokines, matrix proteins, and other elements. It is likely that the microenvironment contributes to the differential expression of genes in the endothelial cells. Due to the prolonged culture period necessary to generate sufficient cells for analysis, it is probable that other genes are differentially expressed in vivo than were able to detect in this investigation. However, the microarray approach yieled information regarding differential gene expression in marrow and metaphyseal vascular endothelial cells. Based on these data, a more in-depth analysis of the identified genes can be pursued at the protein level using in situ immunohistochemical techniques.

ACKNOWLEDGMENTS

We thank Virginia Gilman for technical assistance and Dr. Craig Praul for his microarray expertise.

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