Journal of Cellular Biochemistry 84:108–114 (2002)

Cellular and Molecular Properties Associated With Osteosarcoma Cells

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Abstract Osteosarcoma cells are recognized by abnormal function that causes a primary bone tumor. Osteosarcoma cells U_2OS and SAOS-2 were analyzed for the expression of cell surface markers. High expression was quantified for hyaloronidase receptor (CD-44) > moderate for integrins (CD-51 and -61), > and lower for selectins (CD-62). High mitotic capacity were demonstrated by gene expression (measured by RT-PCR) and the protein level (measured by FACS) for cFOS, cMYC, and cJUN. The basic definition of osteosarcoma is excessive production of pathological osteoid. Expression of mRNA for matrix genes osteocalcin, osteonectin, and biglycan was studied. Osteocalcin and osteonectin were detected in RNA from primary cultured marrow stromal, trabecular bone cells, and osteosarcoma cell lines (U_2OS, SAOS-2). mRNA for biglycan was detected only in primary cells and MG-63 cell line and was undetectable in RNA from U_2OS, SAOS-2 osteosarcoma cell lines and by RNA extracted from bone biopsies of osteosarcoma patients. The absence of biglycan message observed in osteosarcoma samples provides evidence for the alterations in the extra cellular matrix which result with non-mineralized osteoid produced by the osteosarcoma cells. J. Cell. Biochem. 84: 108–114, 2002.

Key words: osteosarcoma; cell surface markers; gene expression; biglycan

Osteosarcoma is the most frequent bone sarcoma in children and adolescents. The disease is naturally recognized by abnormal cell proliferation and function. The osteoblasts produce osteoid, a complex of extracellular matrix (ECM) proteins that are normally mineralized. Malignant transformation of osteoprogenitors leading to osteosarcoma is defined by the production of extensive, incompletely mineralized matrix that is visualized by conventional histology staining. Differential diagnosis used the osteoid as the microscopic hallmark to define the pathological features of the tumors [Unni, 1998]. The physicochemical features and molecular alterations of the osteosarcoma matrix are not well characterized. Abnormal ECM content changes the microenvironment and affects the osteoprogenitors signaling, their function and the cells final differentiation. For instance, the changes in matrix component affect the production of matrix metallo-proteinases (MMPs) by tumor cells and cause disruption of the integrity of the ECM [Kido et al., 1999]. Little is known about the alteration in cell proliferation and gene expression during osteoblast maturation that leads to the tumorogenesis of osteosarcoma. Identification of the changes from normal osteoblasts function may provide a better approach for the differential diagnosis of bone tumors.

Our aim was to characterize osteosarcoma cells on the molecular and cellular level. Osteosarcoma cell lines were studied for the expression of cell cycle regulatory proteins, surface markers and messages for several ECM proteins. Alteration in expression of matrix protein was studied in osteosarcoma

Grant sponsor: The Basic Research Fund, Tel Aviv University to D.B.; Grant sponsor: USA-Middle East Cancer Consortium to D.B.; Grant sponsor: Ministry of Health # 4246.

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Received 22 November 2000; Accepted 1 March 2001

DOI 10.1002/jcb.1270

biopsies from patients. This study will lead to a better understanding of the malignant behavior of bone tumors and identification of the changes associated with tumor state.

MATERIAL AND METHODS

Cultured Cells and Biopsies

Osteosarcoma cells lines (U_2OS, SAOS-2, and MG-63), primary marrow stromal cells (MSC) and trabecular bone cells (TBC) were cultured in growth medium Dulbecco's Modified Essential Medium (DMEM) with the addition of 10% heat-inactivated fetal calf serum (FCS). Biopsies from patients were diagnosed for osteogenic sarcoma (age 16–25, Sourasky Tel Aviv Medical Center).

Hormones

17-beta estradiol of tissue culture grade was purchased from Sigma.

Attachment Assay

Sterile cover slips were incubated with 100 μl of Collagen type I (0.05 μg/μl), or bovine serum albumin (BSA) (0.5 μg/μl) (Sigma) dissolved in double distilled water (DDW), then blocked with 1% BSA in phosphate buffered saline (PBS). U_2OS cells were EDTA released and prepared as single-cell suspension at a concentration of 5×10^5 cells/ml in DMEM supplemented with 2% FCS. 200 μl of cell suspension was plated on precoated cover slips and allowed to incubate for 5 h (37°C, 10% CO_2). The cells were fixed in 4% formaldehyde and 5% sucrose in PBS for 20 min, stained with hematoxylin-eosin and examined using light microscopy.

Flow Cytometric Analysis (FACS)

Osteosarcoma cells, U_2OS and SAOS-2, were EDTA-released from cultures, the single cell suspension was analyzed by FACS. The staining for surface antigens employed biotin conjugated antibodies to CD-44, CD-51, CD-61, CD62P, CD62L, and CD62E (Pharmingen). Cell cycle markers were analyzed with antibodies: KI-67 (Dako), cFOS, cMYC, and cJUN (Oncogene) using intracellular staining procedure. Negative control for intracellular staining employed the isotype-matched IgG (mouse IgG1 and rabbit IgG) at the same concentration as the antibody of interest. Secondary antibodies used were Extravidine-PE (Sigma) or FITC-conjugated-anti-rabbit (Jackson Immune Research Laboratories). FACS staining was performed according technical protocols (http://www.pharmingen.com). For each antibody we used 1×10^6 cells for staining procedure. Finally 1×10^4 cells were quantified and the analysis was performed using software from Becton Dickinson.

Molecular Characterization

Cultured cells and biopsies were used for RNA and DNA extraction. RNA was extracted from cell lines and bone biopsies from patients using EZ-RNA kit (Biological Industries, Beth Haemek, Israel) to analyze gene expression. cDNA was generated by reverse transcription with myeloblastosis reverse transcriptase (AMV-RT) and oligo (dT). The same template was used for a series of polymerase chain reactions (PCR) (Takara Shuzo Co. Ltd., Japan). Genomic DNA was extracted using Promega's Wizard purification kit. Gene specific primers used for PCR amplification are detailed in Table I. The PCR products were separated by electrophoresis in 1% agarose gels (SeaKemGTG, FMC), run in Tris Borate EDTA (TBE) buffer and visualized after ethidium bromide staining using imaging system (Bio Imaging System, BIS 202D).

RESULTS

Malignant bone tumor is characterized by the presence of undifferentiated osteoprogenitors that proliferate and develop a mass of tumor tissue recognized as osteogenic sarcoma. We followed the cell adhesion potential of osteosarcoma cells and the expression of surface markers. Cell's attachment to ECM is a prerequisite condition for their activity. The U_2OS cells were used to study attachment to collagen-I, the major ECM protein present in bone matrix, in comparison to non-adherent protein, BSA. U_2OS cells were plated on pretreated glass slides and 5 h later the cells morphology on collagen were distinguishably different from those cultured on BSA. The cells plated on collagen had visible cytoplasmic processes that extended from cell body (Fig. 1A). This is in contrast to cells plated on slides treated with BSA that were not spread out and only the nuclei were observed (Fig. 1B). Our findings clearly demonstrated the adherence capability of U_2OS to collagen I. Next we analyzed the expression of cell surface receptors that affect adherence processes. We quantified the level of

TABLE I. Gene Expression was Detected by PCR

Gene expression	Sequence	References
G3PDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	Clontech, 5405-1
Transcription factors		
c-Fos	AAGGAGAATCCGAAGGGAAAGGAATAAGATGGCT AGACGAAGGAAGACGTGTAAGCAGTGCAGCT	Clontech, 5450-1
c-Myc	TACCCTCTCAACGACAGCAGCTCGCCCAACTCCT	Clontech, 5451-1
c-Jun	TCTTGACATTCTCCTCGGTGTCCGAGGACCT GCATGAGGAACCGCATCGCTGCCTCCAAGT GCGACCAAGTCCTTCCCACTCGTGCACACT	Clontech, 5452-1
Cytokines		
IL-6	CCTCGACGGCATCTCAGC GCAGAATGAGATGAGTTGTC	Yasukawa et al. [1987]
IL-11	ACTGCTGCTGCTGAAGACTCGGCTGTGA ATGGGGAAGAGCCAGGGCAGAAGTCTGT	Clontech, 5927-1
M-CSF	GGCCATGAGAGGCAGTCCGAGGG CACTGGCAGTCCCACCTGTCTGTC	Stratagene, 302029
Extracellular matrix		
Biglycan	GCAGAACAACGACATCTCCG GTTGTAGTAGGCCCGCTTCA GGAAGCTTCGGGCGCTGACAC CCAGGTCGGAGCACTGAACCAC	Fisher et al. [1989]
Osteonectin	TGGATCTTCTTTCTCCTTT TTCTGCTTCTCAGTCAGA	Young et al. [1990]
Osteocalcin	CATGAGAGCCCTCACA CAGATCCCACAGCGAGA	Rickard et al. [1996]

expression of CD-44 a hyaluronic acid receptor, CD-61 and CD-51 subset of integrin proteins and CD62P, CD62L, and CD62E subset of the selectin proteins. Two-osteosarcoma cell lines [U_2OS and SaOS-2] were analyzed for surface molecules expression using FACS (Fig. 2). Ninety seven percent of U_2OS cells and 71% of SAOS-2 cells were positively stained for CD-44 (Fig. 2). CD-51 was expressed by 10 or 30% and CD-61 by 65 or 24% of the U2OS and SAOS-2 cells respectively. Approximately 20% of stained cells were positive for CD62P, CD62L, and CD62E (Fig. 2).

The osteosarcoma cells posses an abnormal regulation of cell cycle which results in high proliferation potential of tumor cells. FACS analysis revealed that 60 to 80% of cells were positively stained for KI-67, c-FOS, c-JUN, and

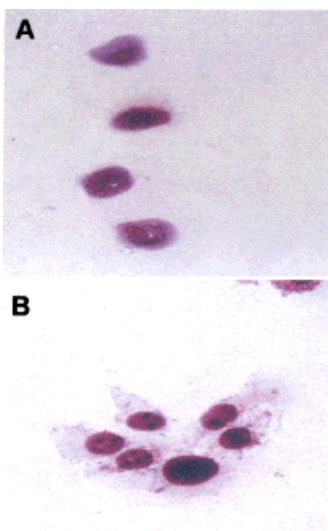

Fig. 1. U_2OS inoculated on precoated glass with BSA (**A**) and Collagen I (**B**). The cells were fixed after 5 h and stained with hematoxylin-eosin. Cells cultured on Collagen I (B) are spread out as compared to the BSA (A) substrata. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

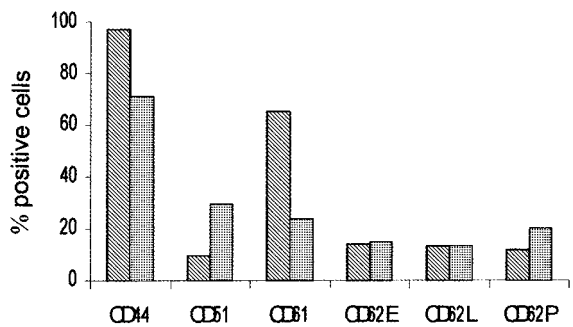

Fig. 2. FACS analysis of surface antigen expression CD-44, -51, -61, -62E, -62L, and -62P in U_2OS and SAOS-2 cells. Histogram summarizes percentage of positive cells, each column represents individual marker (U_2OS, left columns and SAOS-2, right columns).

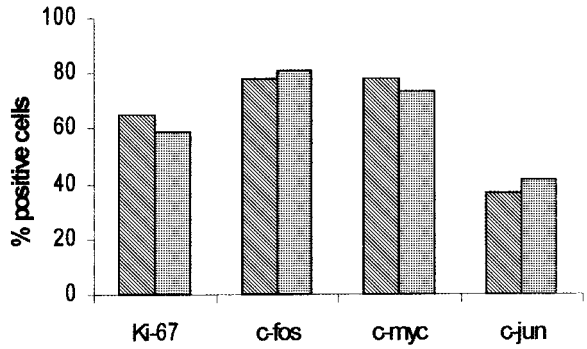

Fig. 3. FACS analysis for transcription regulatory factors KI-67, c-Fos, c-Myc, and c-Jun expressed by U_2OS and SAOS-2 cells. Histogram summarizes percentage of positive cells, each column represents individual protein (U_2OS, left columns and SAOS-2, right columns).

c-MYC proteins (Fig. 3). Complementary, molecular analysis of the RNA messages for c-FOS, c-JUN, and c-MYC from U_2OS and SaOS-2 cells was performed in comparison to normal osteoblasts from MSC and TBC cultures (Table II and Fig. 4). Osteosarcoma cell lines as well as primary cultured cells expressed messages for these genes. Furthermore, analysis was performed for series of genes for cytokines (IL-6, IL-11, and M-CSF) and ECM (biglycan, osteocalcin, osteonectin) using RT-PCR. The messages were detected in all cell types with the exception of biglycan mRNA (Table II and Fig. 4). The biglycan message was expressed only by primary cells (MSC and TBC) and MG-63 and was undetectable in osteosarcoma (U_2OS-2, SAOS) cells (Table II and Fig. 4). The message for biglycan was also analyzed following hormonal challenge with 10^{-8} M 17-beta-Estradiol for 24, 48, and 72 h on U2OS cells. The treatments did not alter the basic level of biglycan expression and the message could not be detected under these conditions. The biglycan expression was studied when amplified the two gene regions: exon 2 (150 bp) and exons 2 to 8 (708 bp) (Table III and Fig. 5). Amplified product of exon 2 from cDNA of primary cells and genomic DNA were of equal size (150 bp) (Table III and Fig. 5). The larger product (exon 2–8) was detected only in cDNA of primary cells. None of the products were detected in cDNA of U_2OS and SAOS-2 cells or in cDNA from a series of bone biopsies from patients diagnosed for OS (Table III and Fig. 5). Histopathological analysis of biopsies from the same patients demonstrates the presence of extensive osteoid (Fig. 6). Typical tumor cells were visualized next to the osteoid, produced by the OS cells that were unmineralized and null for biglycan mRNA expression. In summary, the biglycan message was not expressed in osteosarcoma U_2OS, SAOS-2 cell lines and bone biopsies of OS patient.

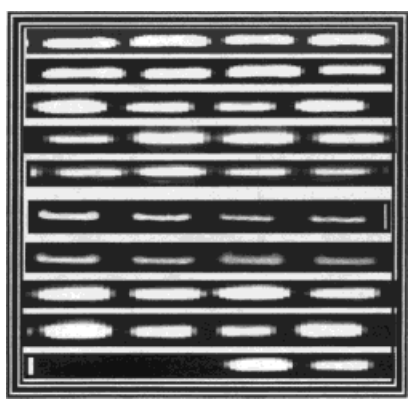

Fig. 4. Gene expression analysis for transcription factors (c-Fos, c-Myc, and c-Jun), cytokines (IL-6, IL-11, M-CSF) ECM (OC, ON, biglycan) and G3PDH in osteosarcoma cell lines and normal primary cultured cells (MSC, TBC). Message is detected for all genes for U2OS, SAOS-2 and MSC and TBC. Biglycan was expressed by MSC and TBC and not by U_2OS and SAOS-2.

DISCUSSION

Osteosarcoma is a malignant solid bone tumor evolved from cells, which possess extended replication. Consequently, the tumor cells become different from the normal osteoblasts at various levels such as cell proliferation and matrix production. Cells reside in a protein network, they secrete and mould ECM to the intercellular space. The ECM exerts profound control over cell's function that is mediated via integrins, a family of cell surface receptors. The

TABLE II. RT-PCR Analyses of RNA from Osteosarcoma Cell Lines (U_2OS, SAOS-2) and Primary Cultured Cells

	Osteosarcoma		Normal cells	
Gene	U_2OS	SaOS-2	TBC	MSC
G3PDH	+	+	+	+
c-Myc	+	+	+	+
c-Jun	+	+	+	+
c-Fos	+	+	+	+
IL-6	+	+	+	+
IL-11	+	+	+	+
M-CSF	+	+	+	+
OC	+	+	+	+
ON	+	+	+	+
Biglycan	–	–	+	+

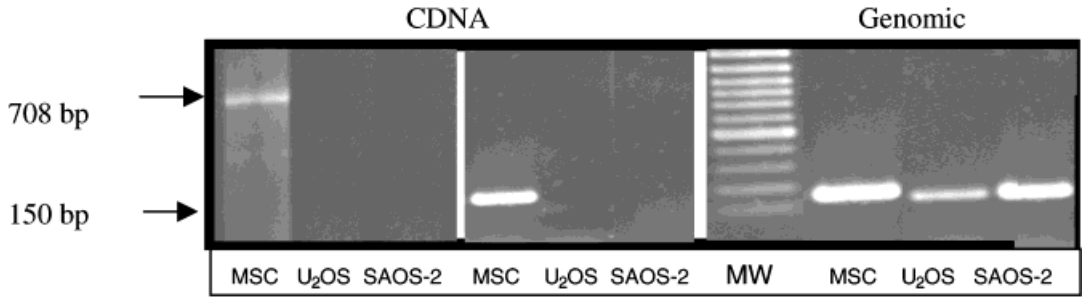

Fig. 5. Analysis for biglycan gene expression by U_2OS, SAOS-2, cDNA and genomic DNA. MSC used as a positive control for gene amplification.

receptors enable cell attachment to the matrix and mediate mechanical and chemical signals from it. Cell surface molecules on osteoblasts [Pittenger et al., 1999] such as integrin mediate cytoskeletal assembly and signals for differentiation. Changes with relevance to the microenvironment constituent are recognized in osteosarcoma. We analyzed osteosarcoma cells [U_2OS and SAOS-2] for cell surface antigens expression. The expression of CD-44 was high in both osteosarcoma cells lines, and was similar to the expression by marrow stroma cells [Pittenger et al., 1999]. The selectin receptors family including CD-62E, CD-62L, and CD-62P were expressed by 20% of the osteosarcoma cells and function in cell–cell interactions. Osteoblasts were analyzed in vivo for the expression of integrin receptors CD-51 and CD-61 [Grzesik and Robey, 1994]. These markers were also expressed in osteosarcoma cells SAOS-2 and U_2OS. The cell surface antigens facilitate cell attachment to an appropriate matrix component. Cell adhesion potential were changed according to their maturational status [Benayahu et al., 1995]. For instance, osteoblasts become increasingly dependent on fibronectin for survival as they mature and differentiate [Globus et al., 1988]. Collagen I is the predominant protein of bone matrix. Demonstrated interactions of U_2OS cells with collagen I revealed that cells cultured on this matrix respond to the ECM, attach and spread-out. Previously it was shown that SAOS-2 cells cultured on collagen-I coated dishes or on a three-dimensional collagen network had increased alkaline phosphatase (ALK-P) activity [Masi et al., 1992]. UMR106-6 cells were cultured on precoated dishes with osteopontin, and demonstrated phosphorylation of focal adhesion kinase (FAK) and increased ALK-P activity, while fibronectin, vitronectin and collagen-I matrices did not induce this enzyme activity [Liu et al., 1997]. It has been reported that focal adhesion kinase is the transducer of survival signals relayed from the matrix [Damsky, 1999]. The role of various matrices on growth and differentiation of MSC was studied at the level of changes in cell morphology, gene expression, and enzymatic activities [ALK-P and CD10/NEP]. It was reported that ECM such as collagen I, osteopontin, and osteonectin had differential effects on osteoblastic MBA-15 cells or 14F1.1 adipocyte cells that are part of the stromal system [Benayahu et al., 1994, 1995; Fried et al., 1996].

TABLE III. Biglycan Gene Expression

Cell type	RT-PCR amplification 708 bp	RT-PCR amplification 150 bp	genomic DNA	G_3PDH
MSC	+	+	+	+
U_2OS	−	−	+	+
SAOS-2	−	−	+	+

Tumor cells including osteosarcoma are recognized by increased proliferation. In U_2OS and SAOS-2 cells expression of c-MYC, c-JUN, and c-FOS at the protein level was analyzed by FACS, and at the molecular level was analyzed by RT-PCR. High levels for c-MYC were demonstrated also on HOS 58 cells [Siggelkow et al., 1998]. The definition of osteosarcoma requires the production of osteoid, which is incompletely mineralized when produced by tumor cells. Histological staining provides visualization of such osteoid for differential diagnosis. Little is known about the molecular components of the osteoid proteins and their physicochemical features. The changes in matrix protein constitution affect the collagen fibril formation. Osteoblastic phenotype is recognized by the

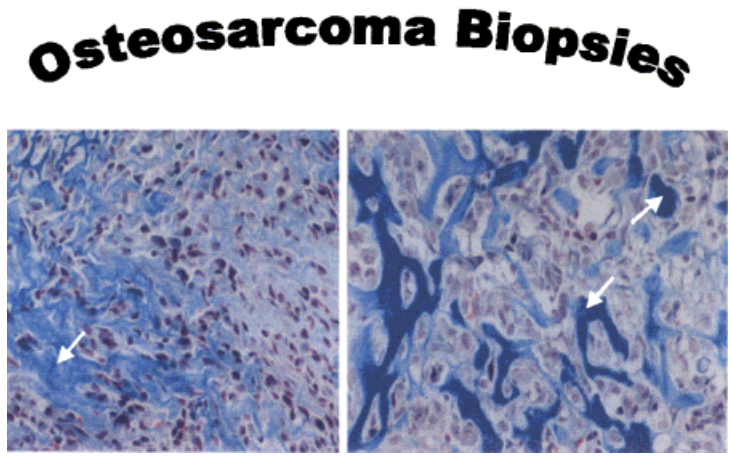

Fig. 6. Histophatology of osteosarcoma biopsies stained by masson trichrom visualized the tumor cells adjacent to incompletely mineralized osteoid (arrow), a characteristic matrix of this tumor. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

coordination of cell maturation with the secretion of specific proteins. These proteins have a pivotal role in modulating cell growth and differentiation and affect the alteration from normal maturation recognized in cancer. We demonstrated the expression of message for matrix proteins, osteocalcin and osteonectin by U_2OS and SAOS-2 osteosarcoma cell lines and osteocalcin expression by HOS 58 was demonstrated elsewhere [Siggelkow et al., 1998]. Down-regulation for fibronectin and thrombospondin by osteosarcoma cells and biopsies was observed using cDNA microarray screening [Wolf et al., 2000]. Cultures of confluent HOS 58 [Siggelkow et al., 1998] and ROS cell lines [Shteyer et al., 1986] produced of matrices typical to osteosarcoma that are improperly mineralized. The ROS cells formed matrix in diffusion chambers in vivo and the assessment for this matrix mineralization capability result with incomplete mineralization [Shteyer et al., 1986]. Bone matrix proteins in OHS-4 osteosarcoma cells exhibited collagen I, osteocalcin, and osteopontin that increased at the message and protein level by application of mechanical load [Harter et al., 1995]. Fibrilles of collagen I interact with proteoglycans that influence the kinetics of fibril formation and the distance between adjacent collagen fibrils. Biglycan is one of the proteoglycans enriched in the bone matrix and is required for matrix organization [Schonherr et al., 1995]. Osteosarcoma cells MG-63 produce pro-collagens that are not representative of the osteoblast lineage but resemble proliferate wound fibroblast [Jukkola et al., 1993]. The proteoglycans decorin and biglycan were detected in dermal fibroblasts and MG-63 cells that were preferentially trapped within the collagen gel in culture [Wegrowski et al., 2000]. These studies refer the MG-63 cells with fibroblastic characteristic rather then other osteosarcoma cells lines. Another study involved with osteoblastic cells lines MC-3T3-E1, MG-63, SAOS-2, and UMR-106 reported the expression of biglycan only by MC-3T3-E1 and MG-63 and showed the effect of TGF-β on regulation of matrix protein synthesis in culture. The osteoblastic cells did not respond uniformly to factors and possessed different levels of TGF-β receptors [Takeuchi et al., 1995]. This data summarize the variations recognized in osteosarcoma cells. It is of prime importance to elucidate the molecular pathways underlining the osteosarcoma alterations. Herein we demonstrated the absence of biglycan message in RNA from osteosarcoma U_2OS and SAOS-2 cells and biopsies from patients diagnosed with osteogenic sarcoma. The absence of biglycan mRNA expression affects the matrix protein constituent produced by the cells and probably leads to defective mineralization of collagen fibrils observed in osteosarcoma.

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