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Differential Gene Expression of Cultured Human Osteoblasts

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Abstract Human cells with osteogenic capacity were studied for differential gene expression. In the first part of the study we compared gene expression of marrow stroma cells (MSC) in comparison to matured osteoblasts cultured from trabecular bone (TBC) that were analyzed by RT-PCR for series of messages. High expression was detected for PTH-r, TGFb1 and biglycan in TBC compared to MSC's. The messages for c-MYC, IL-6, IL-11, M-CSF, osteonectin, and osteocalcin were expressed at the same level in the two populations of cells. In the second part of the study, we analyzed gene expression within the MSC derived from 25 donors (2.5–49 years old) with respect to donors' age and gender. Increased message levels for M-CSF and biglycan were measured in correlation with age of the donors. Gender differences did not affect the expression of cytokines studied (IL-6, IL-11, MCSF, TGFb1). We investigated the effect of Dexamethasone treatment on MSC and monitored an increased expression of IL-11, M-CSF, biglycan, and osteocalcin messages. This study employs primary cell systems (MSC and TBC) to illustrate differential gene expression by osteoblastic cells. The expression was correlated with maturation status of the cells with respect to differences between donors. J. Cell. Biochem. 83: 547–553, 2001.

Key words: osteoblastic cells; marrow stromal cells; gene expression; age/gender'

The variation in skeletal structure based on age and gender can be quantified by size, shape, strength, and mass of bone. In spite of the fact that age- and gender- skeletal differences are recognized in vivo, the regulation processes of the cells that function in maintaining bone tissue are not well established. The osteogenic cells are part of the mesenchymal stromal compartment that contains heterogeneous mixture of cells capable to differentiate into distinctive cell types [Friedenstein et al., 1978; Owen, 1988; Benayahu et al., 1989; Aubin and Fina, 1996; Benayahu, 2000; Kuznetsov and Robey, 2000]. The stromal compartment supports the growth of hemopoietic cells and coordinates the skeletal cells function throughout life. The cells differentiation is regulated and their activity may be altered upon physiological state of the individual. Age related hormonal changes, for example a decline in sex hormones levels, are associated with a decrease in the number and activity of osteogenic cells and an increase in numbers of adipocytes [Kahn et al., 1995; Bergman et al., 1996; Oreffo et al., 1998; D'lppolito et al., 1999; Katzburg et al., 1999; Nishida et al., 1999]. Such changes are recognized in elderly individuals making it difficult to obtain osteogenic cells from the marrow stroma. To study osteoblasts from these individuals, bone fragments from corrective orthopedic surgery are collected and following enzymatic digestion of bone pieces the trabecular bone cells (TBC) are cultivated in vitro. Cultured TBC are used as a model to study mature osteoblasts [Robey and Termine, 1985; Marie et al., 1989; Nolan et al., 1992]. Osteoprogenitors can be isolated from the marrow stromal cells (MSC) and mature osteoblasts derived from trabecular bone (TBC). In this study, we analyzed the gene expression that govern the function of osteoprogenitors and

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mature osteoblasts using two model systems MSC and TBC. The cells derived from various human donors were quantified for the message levels of series of genes. Additionally, we analyzed the differential gene expression of MSC in correlation with age and gender among various donors. The differential expression of substantial markers play an important role in understanding of osteoblastic cell function and their stage of differentiation.

MATERIAL AND METHODS

In Vitro Culture

Human bone marrow stromal cells (MSC) were collected from surgical aspirates of bone marrow (normal donors at age 2.7–49 years) to prepare ex vivo culture. In some experiments the growth medium was supplemented with 10^{-8} M dexamethasone (Dex) (Ikapharm, Israel) and 10^{-4} M L-ascorbic acid phosphate magnesium salt (Sigma, Israel). Human trabecular bone cultures (TBC) were established from bone explants from corrective orthopaedic surgery (donors at ages 60–80 years old). Isolated cells were cultured in Dulbecco's Modified Essential Medium (DMEM) with the addition of 10% heat-inactivated fetal calf serum (FCS). All the experiments were performed using cells from the first to second passage at 80% confluence.

Gene Expression Analysis

Total RNA was extracted from cultured cells (EZ RNA kit, Biological industries, Bet-Haemek, Israel). The RNA was reverse transcripted using avian myeloblastosis virus reverse transcriptase (AMV-RT) and oligo-dT, in order to generate cDNA that served as a template for further polymerase chain reaction (PCR) (Takara Shuzo Co. Ltd., Japan) amplification of specific genes. The integrity of the RNA, the efficiency of RT reaction and the quality of cDNA subjected to the RT-PCR was controlled by amplification of transcript of Glucose-3-Phosphate Dehydrogenase (G3PDH). cDNA amplified from each donor was used for amplification of series of genes. PCR analyses used specific primers for cMYC transcription factor (TF), parathyroid hormone receptor (PTH-R), cytokines (IL-6, IL-11, M-CSF, and TGFb1) and ECM proteins (biglycan, osteonectin (ON), osteocalcin (OC) (Table I). The reaction products were separated by electrophoresis in 1% agarose gels (SeaKem GTG, FMC, USA) in Tris-Borate-EDTA (TBE) buffer. The amplified DNA fragments were stained by ethidium bromide, the optical density (OD) was measured by densitometry (Bio Imaging System, BIS 202D) and analyzed using "TINA" software. The quantification for each gene expression was amplified by PCR at least twice. Semi-quantitative analyses presented comparison of OD of

TABLE I. Primers for Analysis of Gene Expression

Gene	Sequence	Product size on CdNA/genomic	Ref
G3PDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	445 bp, same size	Clontech
PTH-R	AGGCCAGCCAGCATAATGGAA TACTCTGAGCACTTGCCCTC	374 bp on cDNA only	Rickard et al. [1996]
c-MYC	TACCCTCTCAACGACAGCAGCTCGCCC AACTCCT TCTTGACATTCTCCTCGGTGTCCGAGGA	479 bp/1854 bp	Clontech
TGFb1	CAGAAATACAGCAACAATTCCTGG TTGCAGTGTGTTATCCGTGCTGTC	186 bp/2683 bp	CLP
IL-6	CCTCGACGGCATCTCAGC GCAGAATGAGATGAGTTGTC	628 bp/3900 bp	Yasukawa et al. [1987]
IL-11	ACTGCTGCTGCTGAAGACTCGGCTGTGA ATGGGGAAGAGCCAGGGCAGAAGTCTG	322 bp, same size	Clontech
M-CSF	GGCCATGAGAGGCAGTCCGAGGG CACTGGCAGTCCCACCTGTCTGTC	225 bp, same size	Stratagene
Biglycan	CAGAACAACGACATCTCCG GTTGTAGTAGGCCCGCTTCA	720 bp on cDNA only	Fisher et al. [1989]
Osteonectin	TGGATCTTCTTTCTCCTTT TTCTGCTTCTCAGTCAGA	551 bp on cDNA only	Young et al. [1990]
Osteocalcin	CATGAGAGCCCTCACA CAGATCCCACAGCGAGA	310 bp on cDNA only	Rickard et al. [1996]

PCR products for each studied gene normalized to OD of co-amplified G3PDH-PCR product.

Statistical Analysis

Statistical analysis of differential gene expression by RT-PCR of cultured cells derived from various donors was performed by ANOVA test. Results were considered significant for $P < 0.05$.

RESULTS

MSC and TBC represent two-model systems of cultured osteoblasts. The MSC were cultivated in vitro from bone marrow and formed an adherent layer of fibroblast like cells (Fig. 1A). These cultures contain cells that can differentiate to various stromal lineages' including osteoprogenitors. The TBC were obtained following enzymatic digestion of bone fragments that enabled the release of mature osteoblasts to the culture (Fig. 1B). In the first part of the study we compared the gene expression between MSC and TBC cultures. These cells were used for RNA extraction followed by reverse transcription to cDNA. Each cDNA obtained from equal amount of total RNA was analyzed for a series of gene expression using specific primers (Table I). The analyzed messages were c-MYC (cell proliferation), PTH receptor (PTH-r), cytokines (IL-6, IL-11, M-CSF, TGFb1) and ECM proteins (biglycan, osteonectin (ON), osteocalcin (OC)). For each PCR product, OD was measured and normalized towards OD of G3PDH product that served a base line for the semi-quantitative analysis. Genomic DNA contamination was excluded by using primers that amplified products of different size on genomic when compared to cDNA (Fig. 2). PCR products of cMYC (475 bp) or TGFb1 (186 bp) on cDNA from different donors (Fig. 2) were 1860 bp and 2680 bp, respectively, when amplified with the same primers on genomic DNA (control). The messages analyzed from MSC and TBC express equal levels for c-MYC transcription factor (Figs. 2 and 3) and was three-fold higher in TBC then MSC for PTH-r (Fig. 3, $P < 0.002$). We further assessed the expression of cytokines known to locally affect hemopoietic and stromal cells differentiation. Message for TGFb1 was 3.5-times higher in TBC in comparison to MSC (Fig. 3, $P < 0.0015$). The expression of IL-6, IL-11, and M-CSF was at an equal level by TBC and MSC (Fig. 3). ECM proteins are the ultimate products of osteoblastic cells and are required for building the skeleton. The expression of messages for ECM proteins was equal for ON and OC in TBC and MSC cultures while biglycan expression was 1.3-times higher by TBC (Fig. 3, $P < 0.04$). In summary, higher levels were detected for the PTH-r, TGFb1, and biglycan messages by TBC in comparison to MSC cells (Fig. 3).

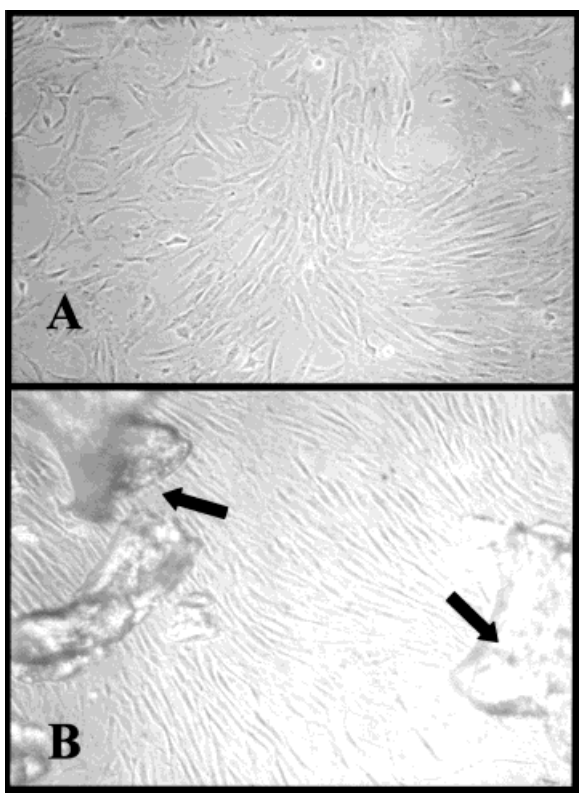

Fig. 1. **(A)** Cultured cells from bone marrow to formed adherent layer of marrow stromal cells (MSC). **(B)** Trabecular bone cells-TBC were obtained following enzymatic digestion of bone fragments (arrow) that enabled the release of cells into cultures.

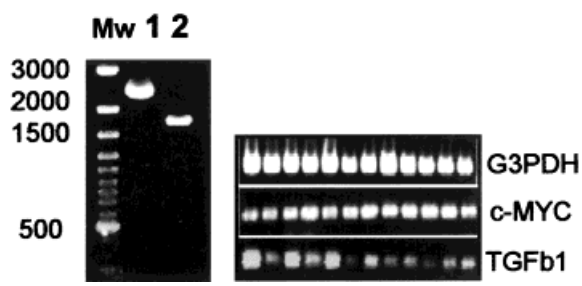

Fig. 2. Genomic DNA used as a template for PCR, left panel represent the products 1 = TGFb1 2680 bp; 2 = cMYC 1860, bp; and right panel is an example for differential expression of G3PDH, c-MYC, and TGFb1 representing different donors.

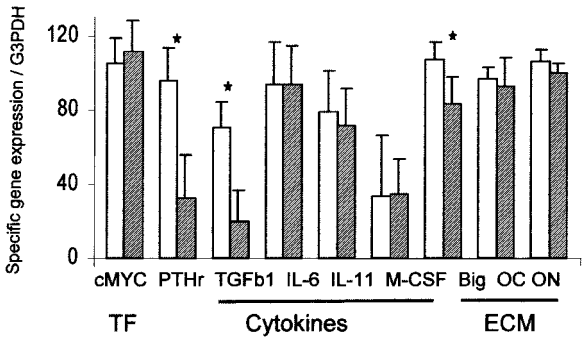

Fig. 3. Gene expression for c-MYC, PTH-r, and cytokines (IL-6, IL-11, M-CSF, TGFb1), ECM proteins (biglycan, OC, ON) was analyzed for cultured TBC (white bars) and MSC (stripped bars) from different donors. Each panel summarizes the average of message level expressed (n = 14) for specific gene and normalized to G3PDH.

The second aim of the study was to explore whether differences in gene expression of cultured MSC could be identified in relation to age or gender. We compared gene expression of cells derived from 25 donors aged from 2.7 to 49 years and some were treated in vitro with Dex. The differential expression was studied for cytokines (IL-6, IL-11, M-CSF), and ECM proteins (biglycan, ON, OC). mRNA for IL-6 was neither affected by Dex treatment, nor by age (Fig. 4a). When cells were Dex treated, they expressed a higher level of mRNA for IL-11 (Fig. 4b) and for M-CSF (Fig. 4c) then untreated cells ($P < 0.049$ and $P < 0.002$, respectively). M-CSF expression was increased in MSC cultures in correlation to the age of donor (Fig. 4c). The mRNA for the analyzed cytokines was not affected by donors' gender. The message for matrix proteins revealed differential expression of biglycan and a higher level was quantified in the Dex treated cells and also with donors' age (Fig. 4d) ($P < 0.013$, $P < 0.000$, respectively).

The OC, which is expressed at late stages of differentiation, was lower then other ECM messages (Fig. 4e) and its expression increased with Dex treatment ($P < 0.009$). The expression of ON was equal between the samples from

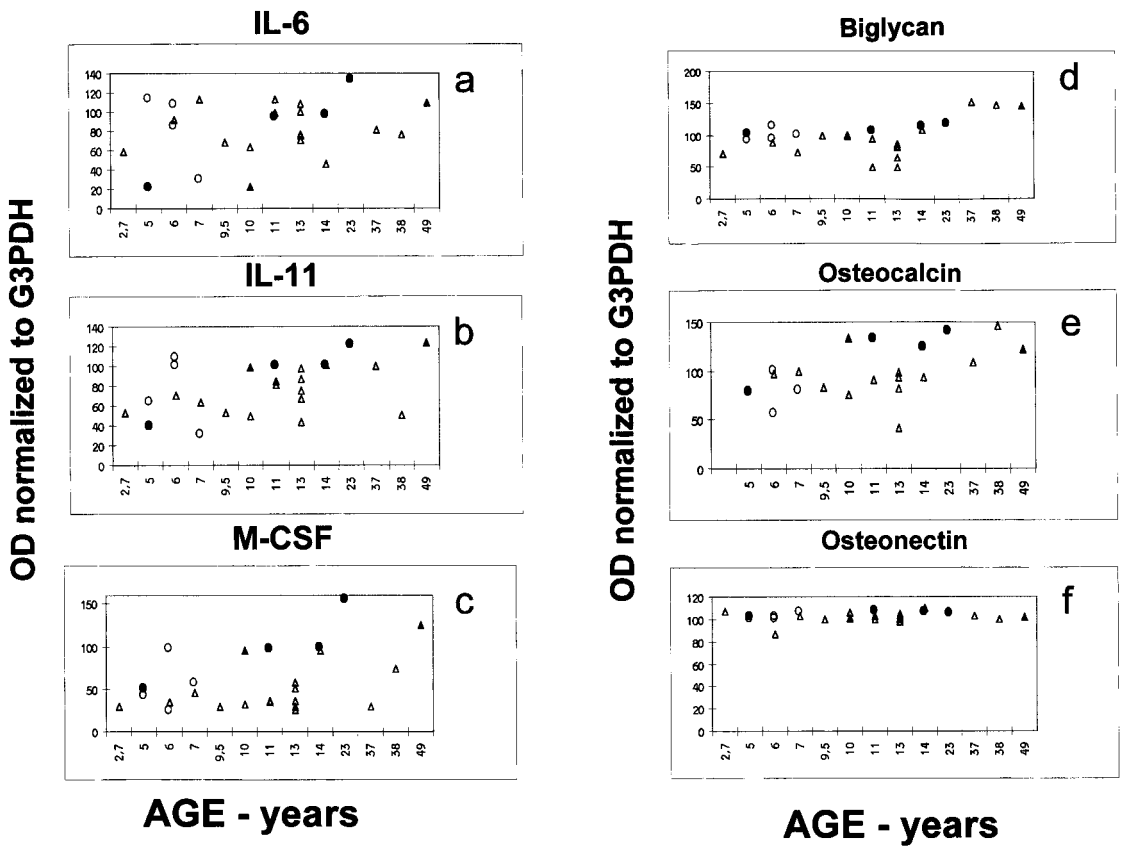

Fig. 4. Gene expression for cytokines (a-IL-6, b-IL-11, c-M-CSF) for ECM proteins (d-biglycan, e-osteocalcin, f-osteonectin) was analyzed for cultured MSC from different donors according to age. Triangle represents male donor and circle female donor. Open symbols are for untreated cells (n = 19) and full symbols represent the Dex treated cells (n = 6). Each panel exhibits the ratio of specific message expression normalized to G3PDH.

various donors and was unaffected by age, gender, or Dex-treatment (Fig. 4f).

DISCUSSION

Cells of the osteoblast lineage control the maintenance of the skeleton formation. Functional cells are at different stages of differentiation and the regulation of osteoprogenitors towards mature osteoblasts is important and is not well characterized. Limited knowledge on the transitional stages of osteoblast differentiation led us to use two model systems represented by different cultured osteoblasts (MSC and TBC). TBC cultures are composed of mature cells liberated from bone explants and matrix that migrated into culture [Robey and Termine, 1985; Marie et al., 1989; Nolan et al., 1992]. MSC are composed of a heterogeneous mixture of cells at various stages of differentiation and with distinct osteogenic potentials [Friedenstein et al., 1978; Owen, 1988; Aubin and Fina, 1996; Benayahu, 2000; Kuznetsov and Robey, 2000]. The use of these models enabled us to quantify expression of the genes activated during osteoblasts maturation. TBC and MSC cultures were assessed for the expression of message for c-MYC, early response growth related transcription factor that was detected in both MSC and TBC cultures. We further compared the cell systems [TBC and MSC] for PTH-r expression and demonstrated higher levels for this message in TBC, as compared to MSC cultures. The importance of PTH action on osteoblasts function as demonstrated on cell–cell communication [Massas and Benayahu, 1998], alkaline phosphatase activity [Benayahu et al., 1995], and matrix-production [McCauley et al., 1996]. Cytokines are involved in bone remodeling, where TGFb is recognized as an important player. TGFb1 regulates osteoblasts activity and is speculated to be involved with osteoblast maturation [Bismar et al., 1999]. A stimulatory effect of TGFb1 on alkaline phosphatase and collagen I expression was more prominent in TBC then MSC cultures [Kassem et al., 2000]. In this study, we demonstrated higher expression of TGFb1 message in cultured TBC then MSC. No differences were observed between TBC and MSC for the expression of cytokines (IL-6, IL-11, and M-CSF). The potential of MSC to secrete cytokines provides cross talk between stromal microenvironment and hematopoietic tissue. It is known that cytokines are altered in relation to age or gender or when individuals are pharmacologically treated. The differential genes expression was therefore studied in MSC harvested from 25 donors in relation to age/gender or Dex treatment in vitro. Dexamethasone is recognized to promote osteogenesis in vitro, and induced the expression of osteogenic markers in MSC [Fried et al., 1993; Fried and Benayahu, 1996; Kuznetsov et al., 1997; Shur et al., 2000]. The expression for IL-6, IL-11, and M-CSF revealed no effect of donor's gender for either cytokine studied. Dex treatment increased the expression of IL-11 and M-CSF mRNA.

M-CSF expression was higher with donor's age, while IL-6 expression was not affected either by treatment or by age. Heterogeneity of MSC causes the complexity and sometimes discrepancy of reports on the levels of cytokine expression and the effects of stimulatory treatment in cultures. It is believed that increased levels of IL-6 are associated with the inhibition of osteoblastic function [Motomura et al., 1998]. Dex suppressed the production of IL-6 and IL-11 by MSC of heterogeneous population of human origin [Haynesworth et al., 1996]. In an earlier study we assessed differential gene expression of clonal MSC in correlation with osteogenesis following in vivo implantation [Kuznetsov et al., 1997]. It was shown that osteogenic MSC expressed higher mRNA levels for IL-6 and IL-11 then nonosteogenic [Shur et al., 2000]. The role of osteogenic compartment of MSC affects the cytokine expression and probably defines the response to in vitro stimulatory treatment. The ultimate characteristic of the differentiation of osteogenic cells is their ability to produce and secrete extra cellular matrix proteins that later mineralizes. The expressions of messages for noncollagenous proteins (osteonectin, osteocalcin, biglycan) were measured. Osteonectin is expressed through the different stages of osteoblastic differentiation, biglycan expression is correlated with higher differentiation and mineralization and osteocalcin is considered to be late marker for osteoblasts. We measured equal levels of osteonectin and osteocalcin RNA by TBC and MSC where these messages did not differ among the MSC from various donors irrespective of age, gender, or treatment. Message for biglycan was significantly higher in TBC cells that probably reflected the more advanced stage of osteoblastic maturation of these cells. In MSC the mRNA

level for biglycan was increased in Dex-treated cells. Clonal MSCs with different osteogenic capacity were analyzed for their expression of matrix proteins. Biglycan message was at a higher level in Dex-treated osteogenic clones compared to treated non-osteogenic clones [Shur et al., 2000]. We demonstrated the absence of message for biglycan in osteosarcoma cell lines and biopsies from patients diagnosed with osteogenic sarcoma [Benayahu et al., 2001]. The osteosarcoma is a mass of proliferating osteoprogenitors cells and the absence of biglycan in osteosarcoma is correlated with existence of nonmineralized osteoid, a pathological diagnostic marker. The biglycan and osteocalcin are expressed by mature osteoblasts. The osteocalcin expression was generally low in all untreated MSCs and increased following Dex treatment which was consistent with reports that glucocorticoid triggers the cells' differentiation along the osteogenic pathway [Subramaniam et al., 1992; Kimoto et al., 1994; Kuznetsov et al., 1997]. Studied patterns of RNA expression allows to address the question of the involvement of each particular gene in the process of osteoblasts maturation that is essential in the maintenance of the skeleton throughout life. These findings extend the knowledge on message expression of genes important for skeletal cell function.

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