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ARTICLES

Analysis of Mesenchymal Cells Derived From an Chondrodysplasia Punctuate Patient and Donors

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Abstract Conradi–Hunermann syndrome (CDPX2) is X-linked dominant disorder appeared with aberrant punctuate calcification. The skeletal cells derived from the marrow stroma are active in maintaining the skeletal formation. We obtained mesenchymal stem cells from a patient with CDPX2 and studied the formation of colony forming unit-fibroblast (CFU-F) in vitro in comparison cells obtained from normal donors. Cultured cells were studied morphologically and subjected to gene expression analysis. Marrow stromal cells (MSC)-chondrodysplasia punctuate (CDP) cells from CDPX2 were identified by their mosaic morphology formed three phenotypically distinct types of CFU-F colonies. One type consisted of normal fibroblasts with developed cell body and cellular processes; the second type contained pathological small cells without processes; and the third type comprised of mixed cells. We compared gene expression by the MSC-CDP to cells from normal donors. Transcription factors analyzed proliferation potential were similar in both normal and mixed colonies of MSC-CDP and similar to normal MSCs. The message expression for cytokines and extra cellular matrix (ECM) proteins revealed similar expression for biglycan, osteocalcin, and osteonectin, while IL-6, IL-11, and M-CSF mRNA levels were significantly higher in normal cells than in MSC-CDP. Mixed cells had elevated levels for IL-6 and M-CSF mRNA, but expressed IL-11 at the normal range. The studied genes were expressed at lower levels by the pathological (MSC-CDP) cells compared to normal ones. Hence, MSC-CDP was demonstrated to display abnormal morphology and transcription of several investigated genes. This study further illuminates the basis of the mosaic pattern of mesenchymal cells derived from a patient affected with CDPX2, and their gene expression involvement. J. Cell. Biochem. 93: 112–119, 2004.

Key words: skeletal dysplasia; Conradi–Hunermann–Happle syndrome; marrow stromal cells

X-linked dominant Conradi–Hunermann syndrome (CDPX2; MIM 302960) is chondrodysplasia punctuate (CDP), recognized as a disorder with aberrant punctuate calcification. This is most prominent around the vertebral column, pelvis, and long bones in CDPX2. Additionally, CDPX2 may show asymmetric rhizomelia, sectional cataracts, patchy alopecia, ichthyoids, and atrophodermia [Happle, 1979]. The phenotype in CDPX2 females ranges from stillborn to mildly affected individuals identified in adulthood. CDPX2 is presumed lethal in males, although few affected males have been reported (OMIM). The genetic background of the disease was identified through mutation detection in the sterol-Δ^8 isomerase gene (*EBP*) [Braverman et al., 1999; Derry et al., 1999], which leads to abnormal metabolism and plasma or tissue sterol profiles. In CDPX2 patients, an increased level of 8-Dehydrocholesterol and 8(9)-cholestenol was detected [Kelly et al., 1999; Becker et al., 2001]. This pattern of accumulated cholesterol intermediates suggests a

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deficiency of 3B-hydroxysteroid-Δ^8, Δ^7-isomerase (sterol-Δ^8 isomerase), which catalyses intermediate step in the conversion of lanosterol to cholesterol. The sterol-Δ^8 isomerase (EBP) has been mapped to Xp11.22-p11.23 and several mutations were identified in CDPX2 patients. The functional significance of two missense alleles was identified through expressing them in a sterol-Δ^8 isomerase deficient yeast.

CDPX2 is characterized by an aberrant punctuated calcification involving the deterioration of skeletal development. The affected patients are identified at the somatic level and are recognized clonally, as reflected in the mosaic appearance of the disease. Such a defect relates to cell differentiation. However, the skeletal cells have not been investigated to date in order to evaluate their function in the disease. The bone marrow stromal cells (MSCs) contain progenitors that give rise to chondrogenic and osteogenic cells [Owen, 1988; Aubin and Fina, 1996; Kuznetsov et al., 1997; Benayahu, 2000; Shur et al., 2002]. Cells that are defective appear in the affected sites in this disorder and the effect is recognized in a colonization pattern. The cells differentiated from mesenchymal stem cells form the colony-forming unit-fibroblast (CFU-F). The differentiation of MSC into functional cells involves coordination and activation of a series of genes. The programmed genes implicated in the regulation of proliferation include transcription factors [Angel and Karin, 1991; McCabe et al., 1996]. Cell differentiation can be studied by examining expression of mRNA for certain cytokines and extra cellular matrix (ECM) proteins. Cytokines play an important role in bone remodeling and the ability to express ECM is a critical aspect of skeletal differentiation. The expression of such genes reflects the stage of cell differentiation along the osteogenic lineage [Benayahu et al., 1994; Cheng et al., 1994; Shur et al., 2001].

In this study, we report on a patient with CDPX2 displaying severe alterations in bone growth and ossification. This study is a first report on the cellular and molecular aspects of MSC obtained from CDPX2 patient. Following cell expansion in culture, we analyzed the cell morphology, growth, and expression of a series of molecular markers involved in bone development and remodeling. We compared the examined parameters with those obtained from cells retrieved from normal donors.

MATERIALS AND METHODS

Patients

Orthopedic background. A 5-month-old female child, born to normal parents with no contributory familial history, was diagnosed with CDPX2. She presented hypotonic delayed motor development, frontal bossing with nasal hypoplasia, sparse hair with circumscribed alopecia, right cataract, and atrophodermia. The child had been in close follow-up for multiple muscular-skeletal manifestations. Her skeletal findings included progressive thoraco-lumbar scoliosis, a short hypoplastic right femur along with a marked leg length discrepancy, coxa vara, coxa brevis, an overriding greater trochanter, dysplastic acetabular changes, and right lateral femoral condyle (Figs. 1 and 2). Incomplete ossification of the right lateral femoral condyle and spine was noted immediately after birth (Fig. 1A1) and continued during growth (Fig. 1C2). Slow rate of growth and ossification of the femoral head and the lateral condyle was observed (Fig. 1) leading to shortness of the right upper femur, which in turn led to length discrepancy of approximately 10 cm (Fig. 2). She currently suffers from lumbar scoliosis and will eventually results with bracing of the spine. An informed consent was obtained from the parents of the patient and from the healthy donors and the Institutional Review Board of the Dana Children's Hospital, Tel-Aviv Medical Center, approved the study.

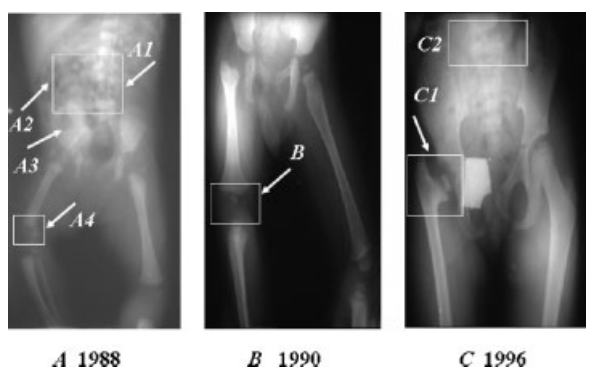

Fig. 1. The X-rays present the progression of the Conradi–Hunermann syndrome (CDPX2) disease. **A**: Five-month-old female child was diagnosed with the disease. Changes are observed in the spine (**A1**), ribs (**A2**), proximal (**A3**), and distal (**A4**) femoral epiphyses, leading to a short femur and leg length discrepancy (**B**). Ossification of the femoral head and distal femur continued at a very slow rate (**C1**) and the changes in the spine (**C2**) led to scoliosis at 8-years-old.

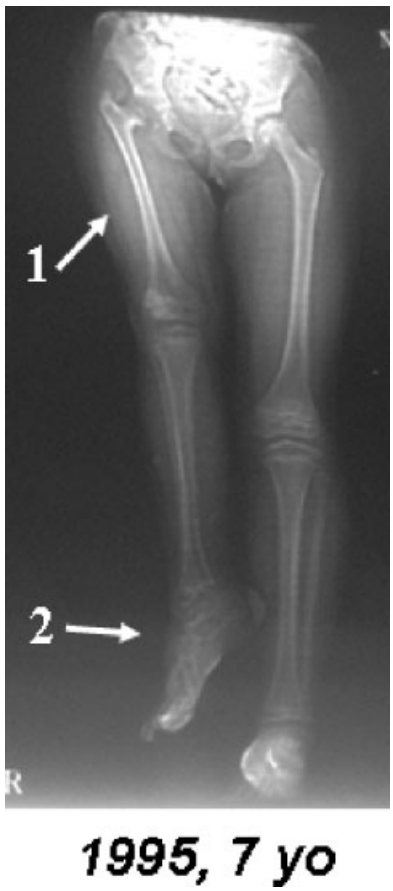

Fig. 2. Leg discrepancy increased to approximately 10 cm as shown in the X-ray.

Biochemistry analysis. Sterols in skin fibroblasts cultured in the absence of cholesterol for 1 week were analyzed by gas chromatography [Kelley, 1995], and revealed markedly increased levels of 8(9)-cholesterol to cholesterol (5.7%; normal = 0.16% [range: 0.04–0.6]). This pattern was consistent with the diagnosis of sterol-Δ^8 isomerase deficiency.

Skeletal cells study in vitro. Bone marrow from the patient at age of 9 years and from eight healthy female donors (age 5–12 years) was collected using surgical aspirates in order to prepare ex-vivo cultures of MSC. Cells were cultured in 75 cm^2 flasks (Falcon, Bedford, MA) containing 20 ml of growth medium, Dulbecco's Modified Essential Medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS). Low-density plating (1.5×10^4 cells/cm^2) was performed to ensure single colony growth (CFU-F). Under these conditions, the hematopoietic cells died and the cultures finally remained only with cells forming the adherent stromal fibroblast layer. The appearance of the fibroblastic cells reflected the proliferation of the stem cells and resulted in different cell types. The primary cells were not passage and colonies were individually collected and pooled for RNA extraction.

Gene expression analysis of MSC. Differences in gene expression between the patient's cells and cells from normal donors were analyzed. A detailed comparison of the patient's gene expression was performed to the mean and range of normal values obtained from healthy donors (n = 8). Differential gene expression was analyzed using total RNA. The extracted RNA (EZ RNA kit, Biological industries, Beit-Haemek, Israel) was then reverse transcriptase using avian myeloblastosis virus reverse transcriptase (AMV-RT) and oligo-dT in order to generate cDNA. This cDNA then served as a template for the polymerase chain reaction (PCR) (Takara Bio Inc., Seta, Japan). For PCR testing, specific primers representing transcription factors, cytokines, and ECM proteins were used (Table I). The reaction products were separated by electrophoresis in 1% agarose gels (FMC, Philadelphia, PA) in Tris Borate EDTA (TBE) buffer. The amplified DNA fragments were visualized by ethidium bromide staining and their optical density (OD) was determined by densitometry (Bio Imaging System, BIS 202D, Preston, Australia). 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). For each donor we used the same cDNA from the RT reaction for amplification of each specific gene. The differential expression for each gene amplified was performed by PCR at least twice. A semi-quantitative analysis for each gene product was normalized by comparison to OD of the co-amplified G3PDH-PCR product.

RESULTS

The defect that affects the bones in CDPX2 is manifested in the aberrant calcification or punctuated calcification characterizing the disease. CDP is characterized by a localized skeletal manifestation, demonstrating an aberrant calcification of the bone in the affected site. This led us to study the cells that differentiated to bone cells from the affected site in the CDPX2 patient. We analyzed the cellular and molecular

TABLE I. Primers Used for Gene Expression Analysis

Gene expression	Sequence	References
G3PDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	Clontech, 5405-1
Transcription factors		
c-Fos	AAGGAGAATCCGAAGGGAAAGGAATAAGATGGCT AGACGAAGGAAGACGTGTAAGCAGTGCAGCT	Clontech, 5450-1
c-Myc	TACCCTCTCAACGACAGCAGCTCGCCCAACTCCT TCTTGACATTCTCCTCGGTGTCCGAGGACCT	Clontech, 5451-1
c-Jun	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	CAGAACAACGACATCTCCG GTTGTAGTAGGCCCGCTTCA	Fisher et al. [1989]
Osteonectin	TGGATCTTCTTTCTCCTTT TTCTGCTTCTCAGTCAGA	Young et al. [1990]
Osteocalcin	CATGAGAGCCCTCACA CAGATCCCACAGCGAGA	Rickard et al. [1996]

properties of the MSC-CDP patient and compared them to MSC from normal donors. Ex-vivo cultures were established from bone marrow cells. The cells were plated at high dilution and form the CFU-F. On day 17 of the primary culture, three types of colonies were identified: colonies with abnormal-pathological cells, colonies with normal fibroblast-like MSC, and mixed colonies (Fig. 3). Cells that appeared to be small and without processes were considered abnormal and pathological (Fig. 3A). The mixed colonies consisted of normal fibroblasts together with abnormal cells (Fig. 3B). These latter colonies were small in size and did not form multi-layers. Normal cells were presented by adherent fibroblasts, which appeared larger and with well-defined cellular processes (Fig. 3C) and formed colonies that were multilayered (Fig. 3D).

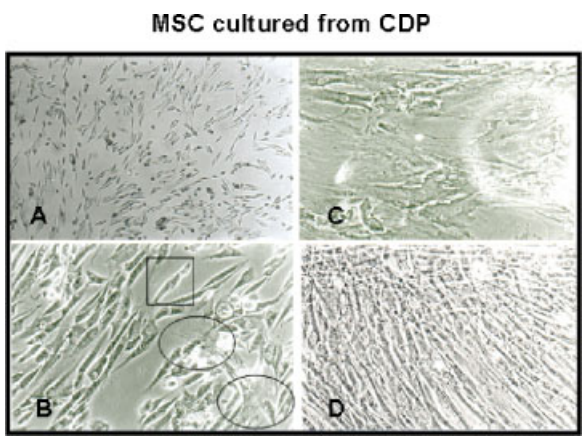

Fig. 3. Morphology of marrow stromal cells (MSC)-chondrodysplasia punctuate (CDP). Cells were plated at high dilution and formed CFU-F identified by three types of colonies. **A**: Pathological cells are small and lacking cell processes. **B**: Mixed colonies, based on cell morphology are normal fibroblastic (in circle) and pathological cells (in box). These two types of colonies were smaller in size and did not form multi-layer. **C**: A normal appearance of fibroblastic cells, large body and cells processes formed large multi-layered colonies with higher number of cells (**D**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

We studied the expression of transcription factors (c-Myc, c-Jun, and c-Fos) by MSC from normal donor cells (Fig. 4A) and MSC-CDP (Fig. 4B). Pathological cells had lower levels of expression than those of the normal population. The normal and mixed colonies from the CDP patient were at normal range of expression for c-jun and c-fos mRNA, while c-myc mRNA expression was slightly lower. The expression of cytokines IL-6, IL-11, and M-CSF was studied for normal cells (Fig. 4C) and for MSC-CDP (Fig. 4D). mRNA levels in the normal colonies of the MSC-CDP were 1.5 to 2.5-fold higher than mean value of the normal group. The cells from mixed colonies expressed mRNA levels in the normal range. The pathological MSC-CDP expressed lower mRNA levels for IL-6, IL-11, and M-CSF compared to mean value of normal MSCs. ECM proteins are the ultimate products of the osteoblasts that are responsible for building the skeleton. Analysis of mRNA expression for byglican, osteocalcin, and osteonectin demonstrated similar expression between all matrix genes in normal donors (Fig. 4E). Levels of ECM expression for the MSC-CDP (normal

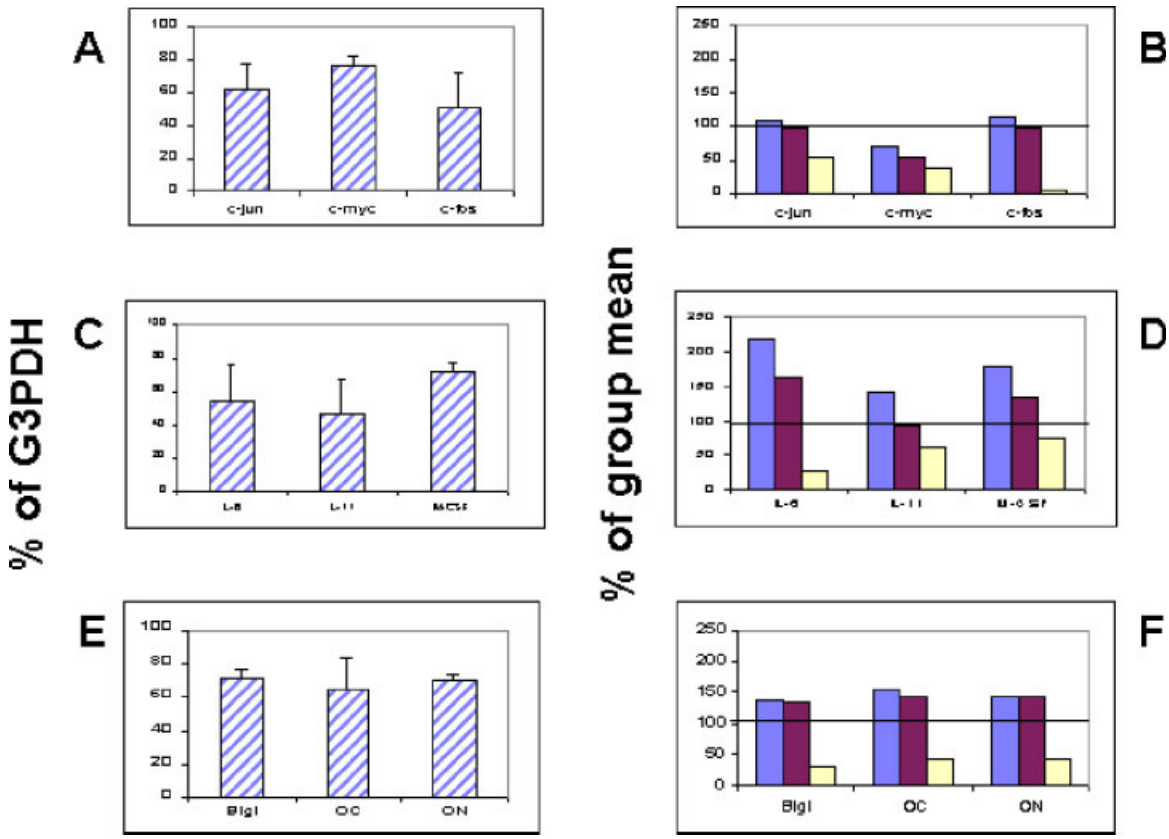

Fig. 4. Gene expression for transcription factors; c-Fos, c-Myc, c-Jun by cells from donors (**A**) and from CDP patient (**B**). Gene expression for cytokines (IL-6, IL-11, M-CSF) by donors (**C**) and CDP patient (**D**). Gene expression for extra cellular matrix (ECM) proteins (biglycan, osteocalcin, osteonectin) from donors (**E**) and from CDP patient (**F**). Panels A, C, E exhibit the ratio of expression for specific messages normalized to glucose-3-phosphate dehydrogenase (G3PDH). Panels B, D, F represent the three cell types cultures (grey, normal colonies; black, mixed colonies; and white, pathological colonies) from CDPX2 patient and mean value of gene expression calculated for normal donors. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and mixed populations) were at the upper normal limit (Fig. 4F) while the pathological MSC-CDP expression was lower than the mean value of the normal group.

In summary, the cells from the diseased site expressed a mosaic pattern of growth and morphology. Moreover, the gene expression that was analyzed emphasized the changes from the normal pathway that underline the manifestation of these cells in the disease.

DISCUSSION

Conradi–Hunermann–Happle syndrome is a rare X-linked dominant disorder characterized by epiphysis stippling and short stature. The lesions in the bone appeared focally within the involved tissue. The characteristic phenotype of CDPX2 is caused by the asymmetric or mosaic appearance of growth abnormalities, stippling, and skin involvements.

In this study, we revealed the morphology of the bone stromal cells derived from the patient's osteotomy has a defective mosaic pattern, as does the gene expression level. The appearance of abnormal, mixed, and normal primary colonies from the stromal bone marrow cells of the patient is explained by lionization in this abnormal regions; the lionization of the normal X allele while in the normal sites the normal allele is active and the mutated allele is lionized. This is consistent with the finding from the patient's mosaic-mixed appearance of MCS growth and phenotypic abnormalities of CDP-patient cells.

The CDP is related locally to mis-calcification of matrix proteoglycans that are produced by osteoblasts such as decorin, biglycan, osteocalcin, and fibromodulin and play a role in bone mineralization. Patients with CDX were studied for polymorphism in several genes and nothing was detected in the decorin gene, while

three variants were identified in the biglycan and one in the fibromodulin gene [Sztrolovics et al., 1994]. In the biglycan gene a variation related to the 5′-untranslated region was identified in one case and at the coding region to the core protein [Das et al., 1994]. Recently, a series of mutations were found in the sterol-Δ^8 isomers emopamil-binding protein (EBP) [Becker et al., 2001]. It was speculated that the deficiency in sterol-Δ^8 isomerase found in CDPX2 causes a sterol toxic effect through the abnormal intermediates of cholesterol metabolism. This may have interfered with the function of cholesterol-modified proteins. The CDPX2 affected patients were identified with skeletal manifestations and the interference in sterol metabolism assumed to play a role in the bone defect. Overlapping developmental phenotypes of skeletal disorders suggest that cholesterol deficiency, or the accumulation of a teratogenic sterol precursor, are pathogenic factors that cause the skeletal cells abnormal function. This may lead to skeletal changes during early weeks of gestation due to abnormalities in hedgehog (HH) class signaling proteins during embryogenesis [Porter et al., 1996; Farese and Herz, 1998; Kelley and Herman, 2001].

The present study set out to analyze MSCs that differentiate to mature cells of the chondrogenic and osetogenic lineages. This is a dynamic process and an incomplete maturation immediately affects the local organization of the marrow compartment, which may result in bone disease. Changes involved in this microenvironment affect the osteoprogenitors locally, and it is important to detect the nature of such changes in order to find new modalities that can restore normal skeletal function. MSC proliferating cells progress through the onset of differentiation events that occur during osteogenesis. We studied the MSC involvement in CDPX2 disease and examined genes expression involved in cell proliferation and function by MSC from normal donors and a CDPX2 patient. Results demonstrated that mRNA levels of transcription factors were steady across the donor populations but showed a lower expression in the pathological cells derived from the patient. Indeed, CFU-F colonies formed from pathological MSC-CDP contained fewer cells in the pathological and mixed colonies compared to the normal ones. The changes in cell morphology and growth of the patient's cells support the clinical observation of the disease associated with a decreased rate of bone formation. This may result from the inhibition of normal cell proliferation by the pathological cells, through the possible accumulation of the intermediate cholesterol metabolites. The few abnormal cells in the affected colonies reflect possible non-random lionization in favor of the normal allele, leading to an increased proportion of normal cells compared to the abnormal ones in the mixed colonies. Local conditions also affect the osteoprogenitor cells, which may be unable to differentiate into mature osteoblasts. Indeed, the IL-6 and IL-11 mRNA were expressed at a lower level in the pathological cells and higher in the "normal and mixed" MSC-CDP, in comparison to cells from normal donors. Change in cytokines level is recognized in another skeletal disease. In fibrous dysplasia, an increased level of IL-6 was detected. The phenomenon was mimicked using transfection with a construct to mutated Gs-alpha protein in MC-3T3-E1 cells [Motomura et al., 1998]. IL-6 has been observed to have an inhibitory effect on ectoblastic development. We described earlier the cytokine expression in correlation to cloned MSC that were defined for their osteogenic differentiation [Shur et al., 2000]. Constitutive and elevated production of IL-6 was quantified in metabolic diseases such as osteoporosis [Manolagas and Jilka, 1995]. Lower levels of IL-6 and IL-11 were quantified in MSC harvested from women receiving estrogen replacement therapy than those from age-matched controls [Cheleuitte et al., 1998]. The over-expression of message levels by the normal and mixed colonies from the CDP patient may indicate compensation for the pathologic cells at the affected lesion site by possible up-regulation gene expressed in favor of the normal cells. This indicates that cytokines play a major role in the microenvironment that controls the MSC differentiation. The alteration in mRNA expression of cytokine at the affected site indicates that these cells are abnormal and thus contribute to the incomplete differentiation of affected cells resulting in a diseased state observed with aberrant punctuate calcification in the CDPX2.

The evidence that CDP patients had abnormalities in cholesterol biosynthesis causes changing in the cell membrane structure of the affected cells. Alteration in the cell membrane may result with integrity of receptor activity. The impairment of cholesterol metabolites in the cell membrane was demonstrated in chang-

ing of HH signaling pathways. Cells with autonomous sterol abnormalities in CDPX-2, are impaired the function of Indian HH (IHH), which is required for vertebrate skeletal development [Chuang and McMahon, 1999]. Such changes in cell signaling affect other functions and we therefore analyzed the expression of genes of the ECM proteins that are the ultimate products of the osteoblasts that are responsible for building the skeleton. Functional integration of cells and the microenvironment were demonstrated in normal and pathological state. The ECM play a pivotal role in establishing functional skeletal cells circuits, and extracellular signals are intrinsic mechanisms that dictate the fate of progenitor cells. The role of matrix proteins was demonstrated in other skeletal conditions, where biglycan is involved in the mineralization process [Fisher et al., 1989; Schonherr et al., 1995], and its absence in osteosarcoma tumors result with osteoid that impaired mineralization [Benayahu et al., 2001]. We have shown earlier that clonal MSCs have higher biglycan expression in osteogenic versus nonosteogenic cells [Shur et al., 2000]. This gene reflects the stage of cell differentiation into a mature cell. Stromal cells differentiates into osteogenic cells is correlated with their ability to express matrix proteins and undergo mineralization. Gene expression for biglycan, osteocalcin, and osteonectin was equal among cells from the normal donors and those from the CDP patient (normal and mixed populations). The pathological MSC-CDP cells expressed significantly lower levels of mRNA for *ECM* genes, meaning that these cells are incomplete differentiated and incapable of producing the ECM needed for normal bone formation. Any shift from the normal pathway finally results in the manifestation of a disease. In the present study, we extend the knowledge of gene expression by normal MSC compared to cells from CDPX2.

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