Characterization of Osteosarcoma Cell Lines MG-63, Saos-2 and U-2 OS in Comparison to Human Osteoblasts

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Abstract. Background: Osteosarcomas are malignant bone tumors consisting of cells with abnormal cellular functions. Although osteosarcoma-derived cells are commonly used for osteoblastic models, the molecular composition of the osteosarcoma extracellular matrix (ECM) is not well characterized. Materials and Methods: We compared three osteosarcoma cell lines (MG-63, Saos-2 and U-2 OS) with normal human osteoblasts by immunocytochemistry. Cellular characteristics were assessed by morphometric analysis and proliferation kinetics. Results: All investigated osteosarcoma cell lines exhibited very heterogeneous labelling profiles and each differed significantly from that of normal osteoblasts. Saos-2 cells revealed the most mature osteoblastic labelling profile while U-2 OS cells were negative for most of the investigated osteoblastic markers. Conclusion: We conclude that each osteosarcoma cell line exhibits a characteristic labelling profile and thus produces a differently composed extracellular matrix. This can be used in attempts to better characterize osteosarcoma, a as well as for their diagnosis.

Osteosarcoma cells are derived form malignant bone tumors. These cells share some osteoblastic features (1), but their chromosomal alterations lead to abnormal molecular and cellular functions (2). Although osteosarcoma-derived cells are commonly used as osteoblastic models, the molecular malfunctions are poorly investigated. In particular, the expression of extracellular matrix proteins contributing to the osteosarcoma-osteoid is altered, leading to histological and immunohistochemical differences in comparison to normal osteoid (3). This may be used as a potential diagnostic approach to better define osteosarcomas (4).

However, the immunohistochemical alterations of the osteosarcoma extracellular matrix as well as alterations in cell proliferation are not well characterized (3). Previous studies have pointed out differences in gene expression profiles of osteosarcoma cell lines and normal osteoblasts (3,5-7), but neither the cellular protein expression nor the molecular composition of the extracellular matrix were analyzed in detail. Investigation at single cell level in vitro, however, is necessary, because most osteosarcoma contain different subpopulations (8).

In order to better characterize well-known osteosarcoma cell lines, we performed an analysis of the immunohistochemical labelling profile and growth properties of the three different osteosarcoma-derived cell lines MG-63, Saos-2, and U-2 OS in comparison to osteoblasts derived from normal human bone.

Materials and Methods

Cells and cell culture. The osteosarcoma cell lines (MG-63, Saos-2, and U-2 OS) were purchased from ATCC (USA) and cultivated, according to the recommendation of the supplier, in DMEM containing 10% FBS, McCoy’s 5a medium containing 15% FBS and McCoy’s 5a medium containing 10% FBS, respectively. Cells were cultured without any stimulatory supplements or vitamins in T25 flasks (Nunc, USA) in a humidified incubator at 37°C, using a standard mixture of 95% air and 5% CO₂. Forty IU/ml of penicillin/streptomycin was added to each medium. The media and antibiotics were purchased from Gibco and FBS was obtained from Sigma-Aldrich Co. (Germany). For passaging, cells were detached with trypsin/EDTA and subsequently replated.
As controls, human osteoblasts from two different donors (female, 63 and 66 years) were purchased from PromoCell GmbH (Germany). According to Kasperk et al. (1997), these cells were originally isolated from samples of human trabecular bone obtained from the trochanteric region during orthopedic surgery (e.g. hip replacements). The osteoblasts were cultured in osteoblast growth medium (PromoCell) supplemented with 10% FBS serum with 50 µg/ml gentamycin and amphotericin B, containing no further vitamins or hormones.

Morphometry. Morphometric analysis was carried out both on adherent cells and cells in suspension after trypsination. The area and diameter of attached cells (n=100) were measured by phase contrast microscopy, using an image analysis system KS-400 3.0 (Zeiss, Germany). The osteoblasts were cultured in osteoblast growth medium (PromoCell) supplemented with 10% FBS serum with 50 µg/ml gentamycin and amphotericin B, containing no further vitamins or hormones.

Cell proliferation assay. For cell proliferation analysis, osteoblasts were seeded at 4 x 10^4 cells/T25 flasks. At days 3, 4, 7, 14 and 21, cells were detached and their number determined. MG-63, Saos-2 and U-2 OS cells were seeded at 2.5 x 10^5 cells/T25 flask to achieve the same extent of cell confluence as for osteoblasts. At days 3, 4, 7, 14 and 21, cells were detached and counted. All assays were performed in triplicate and the early log-phase was used to estimate the population-doubling-time.

Statistical analysis. For statistical analysis, the arithmetic means, standard deviations and 95% confidence intervals were calculated. The results are presented as mean values ± 95% confidence intervals (Figures 1,2) or mean values ± standard deviation (Figure 3). Differences were regarded as statistically significant (p<0.05) when 95% confidence intervals did not overlap.

Immunocytochemistry. For immunocytochemistry, cells were seeded on glass slides at two stages of cell confluence (50% and 100%). Cells were fixed in methanol containing 0.3% hydrogen peroxide for 20 min and subsequently washed in phosphate-buffered saline (PBS). No enzyme treatment was performed. Non-specific binding of the secondary antibody was reduced with an appropriate serum block. A panel of 19 primary antibodies was applied (Table I) and antibody binding was detected with a Vectastain ABC 'Elite' avidin/biotin/peroxidase kit (Vector Laboratories, USA). In order to allow the detection of low levels of positive immunocytochemical labelling, no nuclear counterstain was used. Non-specific binding of antibodies was controlled by omitting the primary antibody. The heterogeneity of the immunocytochemical results was assessed by a three-point scoring system as described in the legend of Table I.

Alkaline phosphatase activity. Alkaline phosphatase (ALP) activity was detected using Alkaline Phosphatase Kit 85 containing Naphtol AS-MX phosphate and fast violet B salt as chromogen (Sigma, St. Louis, USA).

Results

Morphometry. The mean size of attached osteosarcoma cells was approximately 1/6 of the size of osteoblasts (Figure 1). Unlike osteoblasts, the osteosarcoma cell lines did not vary

Figure 1. Size of adherent cells. Mean cell diameter of adherent cells. Vertical bars represent 95% confidence intervals.

Figure 2. Size of cells in suspension. Mean cell volume of cells in suspension, detached at subconfluence (approximately 50% confluence) and at full confluence (100%). Only osteoblasts show significant differences in cell volume between subconfluent and confluent cells. Vertical bars represent 95% confidence intervals.

Figure 3. Proliferation kinetics. Proliferation kinetics of the investigated cells over a period of 21 days. Osteoblasts seeded at 1,600 cells / cm² reached their saturation density on day 7 at approximately 10,000 cells / cm². Among the osteosarcoma cells, the saturation densities of U-2 OS and Saos-2 differ significantly. Vertical bars represent standard deviation.
their cell size dependent on cell density (Figure 2): no statistically significant difference in cell size could be determined between subconfluent and confluent cells in suspension. Attached osteosarcoma cells revealed a different morphology when compared to osteoblasts. MG-63 cells were oval to spindle-shaped, without branching cell processes. Saos-2 cells were polygonal with few small cell processes, while U-2 OS cells showed a spindle to triangle-shaped form. In contrast, attached osteoblasts exhibited a varying, but nevertheless characteristic, cell shape with several prominent, elongated cell processes.

Cell proliferation assay. The growth characteristics (doubling-time and saturation cell density) of osteosarcoma cell lines differed significantly from that of osteoblasts. All osteosarcoma cell lines revealed a 2 to 3-fold greater mean doubling-time and a 15 to 20-fold higher saturation density than osteoblasts (Figure 3).

Immunocytochemistry. The labelling patterns of all osteosarcoma cell lines were inhomogeneous, i.e. varying proportions of cells within each cell line showed positive and negative reactions for the same antibody (Figure 4, Table I).

In MG-63 cells, only small subpopulations were positive for the osteoblastic markers OC, BSP and decorin. MMP-9 was positive in the majority of MG-63 cells.

U-2 OS cells were negative for the osteoblastic markers OC and decorin. Less than 50% of the cells were positive for collagen type I. Positive labelling was also found for molecules related to cartilage such as collagen types II, IX and X, and for collagen IV.

The labelling results of all investigated osteosarcoma-derived cells were consistent and did not depend on passage number or cell density. In contrast, human osteoblasts changed their labelling profile dependent on cell density. While proliferating osteoblasts in subconfluence showed negative labelling for decorin and osteocalcin, confluent osteoblasts, especially those organized in clusters, were positive for both markers. Labelling for other osteoblastic markers like BSP, osteopontin (OPN), osteonectin (ON), or osteoprotegerin (OPG), or collagen types I and III was consistently positive in osteoblasts (Figure 5, Table I).

Alkaline phosphatase activity. All Saos-2 cells revealed ALP activity, whereas no ALP activity could be detected in MG-63 and U-2 OS cells. Human osteoblasts exhibited a varying ALP activity which was dependent on cell density. In a confluent cell layer approximately 30% of cells showed positive ALP activity, while in subconfluent osteoblasts the number of positive cells was smaller.
Discussion

The aim of this study was the characterization of osteosarcoma-derived cells MG-63, Saos-2, and U-2 OS and the detection of extracellular matrix products in vitro. The analysis of altered osteosarcoma-osteoid in comparison to normal bone may contribute to a better understanding of the behavior of bone tumors and their diagnosis (4,8).

Although osteosarcoma cells share some features of osteoblasts, they differ in many aspects (9), in particular concerning their proliferation kinetics and the osteoid production (10). However, the latter is only poorly investigated (3).

The immunocytochemical analysis of the extracellular matrix proteins contributing to the osteosarcoma-osteoid revealed unique labelling results for each osteosarcoma-derived cell line. The most prominent alterations were the positive labelling results for MMP-9 and collagen-X, which could be detected in all osteosarcoma cell lines but not in normal osteoblasts. Although MMP-9 is expressed during rat osteogenesis (11), it is not found in human osteoblasts (12), an observation confirmed in this study. Each of the three investigated osteosarcoma cell lines revealed labelling profiles with different signs of osteoblastic maturity, but all differed significantly from the labelling profile of osteoblasts. Saos-2 cells exhibited the most mature osteoblastic phenotype with positive results for ALP, OC, BSP, decorin and collagen I and III. However, labelling for ALP was positive in almost every cell, while the number of osteoblasts exhibiting ALP activity was much lower, which is consistent with other studies.

Figure 4. Immunocytochemical labelling results of osteosarcoma cells. Immunolabelling characteristics of MG-63 cells (a,d,g), Saos-2 cells (b,e,h) and U-2 OS cells (c,f,i). (a) MG-63 cells labelled for type I collagen, score = 2 (Table II). (b) Saos-2 cells labelled for type III collagen; score = 1. (c) U-2 OS cells labelled for type I collagen; score = 1. (d) MG-63 cells labelled for MMP-9 in confluence; score = 2. (e) Saos-2 cells labelled for alkaline phosphatase; score = 3. (f) U-2 OS cells labelled for type II collagen. (g) Only a subpopulation of MG-63 was positive for the osteoblastic marker osteocalcin, score=1. (h) The majority of Saos-2 cells were positive for the osteoblastic marker decorin, score=2. (i) U-2 OS cells labelled for type X collagen, score = 1. All bars = 50 μm.
Differences were also found for collagen types III and IV. While only small subpopulations of Saos-2 cells were positive for collagen III, osteoblasts labelled consistently positive for this protein. For collagen IV the ratio was vice versa: only a very few osteoblasts were positive for collagen type IV, in contrast to the majority of Saos-2 cells. The labelling profile of MG-63 cells revealed both mature and immature osteoblastic features and was the most heterogeneous of the investigated osteosarcoma cell lines. While Saos-2 cells represent a mature phenotype, U-2 OS cells were negative for almost all osteoblastic markers but positive for cartilage markers like collagen II, IX and X. In addition, U-2 OS cells showed positive results for type IV collagen, which is only expressed by very early differentiation stages but not by osteoblasts (15,16). Therefore, the U-2 OS cell line is not consistently classified as osteoblastic, but also as fibroblastic (17).

Another difference between the investigated osteosarcoma cell lines and normal osteoblasts was the lack of cell density-dependent morphological changes (18). Contrary to osteosarcoma cells, osteoblasts changed their labelling pattern dependent on cell density. Our results are consistent with that of other groups demonstrating the effect of cell density on osteocalcin (19) and decorin expression (20) as well as ALP activity (21). All osteosarcoma cell lines lack physiological features such as contact inhibition; consequently, cell growth of osteosarcoma cell in cell culture is not restricted to monolayers. The severely altered cellular functions like cell-to-cell communication are typical of the malignant nature of the osteosarcoma cells.

Our results further show that labelling of osteosarcoma-derived cells is more heterogeneous compared to human osteoblasts for almost every protein investigated. This demonstrates the importance of examination techniques at
single cell level and further emphasizes the limits of investigation techniques that require lysis of cell aliquots and homogenization of their mRNA transcripts. Although RT-PCR phenotyping has been extensively performed for MG-63, Saos-2 and U-2 OS cell lines (7,22), these studies have not addressed what proportions of cells produce the transcripts and whether subpopulations exist.

In summary, we conclude that an immunocytochemical labelling profile of extracellular matrix proteins obtained from osteosarcoma cells in vitro can be used in attempts to better characterize osteosarcoma cell lines and to discriminate between different bone tumors and normal bone.

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References