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Biochemical and Biophysical Research Communications 333 (2005) 216–222
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Phenotypic instability of Saos-2 cells in long-term culture

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Received 11 May 2005
Available online 31 May 2005

Abstract

The human osteosarcoma cell line Saos-2 is widely used as a model system for human osteoblastic cells, though its phenotypic stability has not been ascertained. We therefore propagated these cells over 100 passages and compared relevant phenotypic properties. In general, higher passage cells exhibited higher proliferation rates and lower specific alkaline phosphatase activities, though mineralization was significantly more pronounced in cultures of late passage cells. Whereas expression of most genes investigated did not vary profoundly, some genes exhibited remarkable differences. Decorin, for instance, that has been discussed as a regulator of proliferation and mineralization, is strongly expressed only in early passage cells, and two receptors for pleiotrophin and midkine exhibited an almost mutually exclusive expression pattern in early and late passage cells, respectively. Our observations indicate that special care is required when results obtained with Saos-2 cells with different culture history are to be compared.

Keywords: Saos-2 cells; Osteoblasts; Cell culture; Phenotype evolution; Mineralization; Decorin; Anaplastic lymphoma kinase

Since their first description in 1975 [1], Saos-2 cells have been widely used as a model system for osteoblastic cells. Promising advantages for using this cell line are (1) its world wide availability, (2) its good and well-documented characterization, (3) the possibility to obtain large amounts of cells in short time, and (4) the fact that Saos-2 cells exhibit the entire differentiation sequence of osteoblastic cells. The latter point, particularly the ability of Saos-2 cells to deposit a mineralization-competent extracellular matrix [2], makes these cells a valuable model for studying events associated with the late osteoblastic differentiation stage in human cells, as nodule formation can usually not be observed in cultures of primary human osteoblasts isolated from spongiosa of adult donors [3]. Furthermore, working with primary osteoblast preparations always bears the possibility that contaminating non-osteoblastic cells contribute to or even dominate the effects under investigation, apart from the fact that the osteoblastic cells by themselves cannot be expected to be homogeneous, for instance with regard to their differentiation stage.

Important known disadvantages of cell lines derived from tumors, on the other hand, are associated with their origin from a pathological tissue of a single individual, raising the possibility that observed effects may be related to the pathological state of the original tumor rather than being representative for the respective untransformed cell, or might represent peculiarities of the individual donor. Indeed, many tumor derived cell lines have been initially established in order to investigate phenomena that are characteristic for the tumor from which the cells have been derived.

Apart from the fact that cells isolated from tumor samples can be expected to be genetically heterogeneous, another possible point of concern arises from observations that established cell lines may be genetically unstable during long-term cultivation [4,5]. Though systematic studies on phenotype evolution are scarce, karyotype evolution has been reported in HMT-3522 cells cultured over 205 passages that was attributed to

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.05.097

genetic instability, and heterogeneity was accompanied by changes in the growth capacities of the cells [5]. Indicative of phenotype evolution, two variants of the monocytic cell line U937 have been described, one being responsive to tumor necrosis factor and the other one being resistant [6]. And even for Saos-2 cells isolated observations on passage-dependent alkaline phosphatase activity [7] and relative responsiveness of adenylyl cyclase to stimulation by parathyroid hormone and forskolin, respectively, have been reported [8].

Based on these observations we wanted to study more systematically the phenotypic stability of Saos-2 cells during long-term culture. To this aim we cultured Saos-2 cells over approximately 100 passages and looked in selected passages for a set of properties relevant for their use as a model system for osteoblastic cells. Here, we describe that although many of the properties studied were not obviously dependent on passage number, some of them exhibited pronounced passage dependence.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and trypsin/EDTA solution were purchased from Biochrom (Berlin, Germany). Ascorbic acid 2-phosphate, β-glycerophosphate, Sigma Fast *p*-nitrophenyl phosphate tablet sets, and alizarin Red-S were from Sigma (Taufkirchen, Germany), and thiazolyl blue tetrazolium bromide was purchased from Fluka (Taufkirchen, Germany). All other chemicals used were of analytical grade. The human osteosarcoma cell line Saos-2 was from the American Type Culture Collection (Rockville, MD).

Cell culture. For propagation of Saos-2 cells, cells were maintained in DMEM containing 44 mM $NaHCO_3$, 2 mM L-glutamine, and 10% FCS (growth medium) at 37 °C in 10% CO_2 in air. After reaching approximately 80% confluency, cells were subcultured by rinsing the cell layer with 0.05% (w/v) trypsin and 0.53 mM EDTA, and subsequent incubation at 37 °C for 10 min. Splitting ratios were approximately 1:5. From every fifth to 10th passage, aliquots were stored in liquid nitrogen in 5% (v/v) dimethyl sulfoxide in DMEM containing 10% FCS for later use in experiments. After propagation over 100 passages, cells from selected passages were thawed and initially plated into 75 cm^2 culture flasks (approximately 1.5×10^4 cells/cm^2) in growth medium. Unless otherwise stated cells were plated for experiments at an initial density of 1.0×10^4 cells/cm^2 and maintained in growth medium for 24 h. Then fresh growth medium containing additionally 0.2 mM ascorbic acid 2-phosphate was added and replaced twice a week. For mineralization studies, cultures were further supplemented with β-glycerophosphate at a final concentration of 10 mM during the last 24 h.

MTT assay. In the proliferation studies, viable cells were determined by the MTT assay that relies on the ability of mitochondrial dehydrogenases to oxidize thiazolyl blue tetrazolium bromide (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to an insoluble blue formazan product. Cells were incubated with growth medium containing 0.3 μM MTT at 37 °C for 1 h. After removing the culture supernatant and washing the cells, isopropanol containing 40 mM HCl was added and the optical density of the solution was read at 530 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader.

Alkaline phosphatase activity. Alkaline phosphatase activity was assayed in cell lysates by determining the release of *p*-nitrophenol from *p*-nitrophenyl phosphate using Sigma Fast *p*-nitrophenyl phosphate tablet sets according to the instructions of the manufacturer. Cells were lysed with 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$, and 0.1% (v/v) Triton X-100 in 20 mM Tris/HCl, pH 7.4, and release of *p*-nitrophenol was determined by measuring the absorbance at 405 nm over a period of 4 min. In parallel triplicate cell cultures, cell numbers were determined using a Casy 1 cell counter (Schärfe System, Reutlingen, Germany), and alkaline phosphatase activity was normalized to cell number.

Matrix mineralization. For quantification of matrix mineralization, cell cultures were stained with alizarin Red-S essentially as described [9]. Cells were washed with PBS at room temperature and fixed with 4% formaldehyde in PBS. Fixed cell cultures were stained with 40 mM alizarin Red-S (pH 4.2) for 10 min using an orbital shaker. Due to the solubility of amorphic mineral deposits at pH 4.2, staining under these acidic conditions is considered to be specific for hydroxyapatite crystals. To remove non-specifically bound stain, cultures were washed five times with deionized water and once with PBS for 15 min at ambient temperature. Bound dye was solubilized in 10 mM sodium phosphate (pH 7.0) containing 10% cetylpyridinium chloride and quantitated spectrophotometrically at 562 nm. Quantitation by this method has a degree of accuracy similar to the quantitation of hydroxyapatite by binding assay [10].

RT-PCR. Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and digested with RNase-free DNase (Qiagen) to remove any contaminating genomic DNA. cDNA synthesis from total RNA was performed with Omniscript reverse transcriptase (Qiagen) using $(dT)_{15}$ (1 μM) and random hexanucleotide primers (5 μM; Roche Diagnostics, Mannheim, Germany) simultaneously. Aliquots of the cDNAs were incubated with HotStarTaq DNA polymerase (Qiagen) and the primers specified in Table 1. All primers were synthesized by MWG Biotech (Munich, Germany) and were of high purity salt-free (HPSF) quality. The polymerase was activated (15 min at 96 °C) and then 32 (unless otherwise stated) cycles (45 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C) were performed on a RoboCycler Gradient 96 (Stratagene, Amsterdam, The Netherlands). Amplification products were visualized by agarose gel electrophoresis after staining with ethidium bromide. Images were acquired using the ImageMaster VDS system (Amersham Biosciences, Freiburg, Germany) and the accompanying software (version 2.0).

Statistical analysis. All experiments were repeated at least twice, and similar results were obtained in all these independent experiments. Quantitative proliferation and mineralization assays were performed in quadruplicate in these independent experiments, and means and standard deviations of typical experiments are shown. Data were analyzed using Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Saos-2 cells from late passages exhibited increased proliferation rates and decreased alkaline phosphatase specific activity

In order to study the phenotypic stability of Saos-2 cells during long-term culture, we propagated Saos-2 cells over more than 100 passages. As under these conditions selection of cells with higher growth capacity might be expected, we compared the proliferation of cells that had been stored frozen upon different times of propagation. It can be seen in Fig. 1 that cells from later passages exhibited higher proliferation rates than early passage cells, the maximal effect being an increase by approximately 65%. Concomitantly, there was a de-