Adhesion, Growth, and Matrix Production by Osteoblasts on Collagen Substra

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Summary. A number of studies have demonstrated the pivotal role of collagen molecules in modulating cell growth and differentiation. In order to analyze the direct effects of collagen type I on the osteoblastic phenotype, we have devised an in vitro culture system for studying the interactions between bovine collagen type I and Saos-2 cells, a human osteoblastic cell line. Saos-2 cells were cultured both on top of collagen-coated culture dishes as well as inside a three-dimensional collagen network. Plating on dishes treated with collagen induced maximal adhesion of Saos-2 cells after 24-hour incubation. Cells cultured on collagen gel matrix expressed about 2.5-fold more alkaline phosphatase when compared with untreated plastic dishes. On collagen-coated dishes the responsiveness of Saos-2 cells to parathyroid hormone was decreased, whereas no modifications were observed in the effect of vasoactive intestinal peptide on these cells. Using a microfluorimetric measurement of DNA, an increase of proliferation was observed in Saos-2 cells cultured on collagen gel. Saos-2 cells were also able to colonize collagen sponges and in this three-dimensional network they were able to synthesize osteocalcin, as assessed both by immunocytochemistry and radioimmunoassay. In this study we have demonstrated that bovine collagen type I exhibits favorable effects on attachment and functional and growth activities of a human osteoblastic cell line, encouraging its use as a bone graft material.

Key words: Osteoblasts - Collagen type I - "On gel" cultures - Collagen sponges - Osteocalcin.

Cells that do not circulate are supported by complex arrangements of relatively stable macromolecules, collectively referred to as the extracellular matrix (ECM). In vitro studies have shown that cell interaction with the ECM induces dramatic effects on cell shape, growth, and functional properties. Collagens form the framework of this complex in most tissues and the most widely studied matrix components because of their abundance and ease of preparation. In some cases the ECM, especially collagen, has been shown to shift the phenotypic cell expression of already differentiated cell types. For example, collagenous bone matrix induces endochondral ossification and hemopoiesis [1], and mammary ductal epithelial cells' reorganization into duct-like tubules [2, 3]. Moreover, endothelial cells reconstruct blood vessels within collagen gel culture [4], disaggregated chondrocytes proliferate in three-dimensional collagen gels forming chondroids [5], avian skeletal muscle cells form highly contractile myotubes in a similar culture system [6], and phenotypic differentiation is observed in vitro with canancular cell processes in osteoblasts upon components of basement membranes [7].

The interstitial type I collagen is a major structural protein involved in the stability of connective tissue architecture, comprising 90% of the organic bone matrix with the remaining 10% consisting of noncollagenous proteins; most of these proteins have been well characterized [8]. In normal life, productive osteoblasts constitute layers of confluent cells with cell processes that extend into bone matrix. Several attempts have been made to develop suitable systems for studying the osteogenic potential of osteoblasts in vitro. Monolayer cultures on plastic surfaces routinely used to model osteoblast metabolism do not precisely reproduce their in vivo environment, forcing the cells to develop in a constrained two-dimensional architecture. In particular, osteoblastic cells in culture do not acquire the ability of mineralizing until the cultures become multilayered [9-12]. Three-dimensional cultures of osteoblast-like cells have been performed both in collagen matrices [13] and by coating the dishes with agarose [14]. In these conditions the cells are encouraged to elaborate a matrix distinct from that of monolayer cultures and closer to that observed in vivo [13, 14]. The use of collagen sponges may offer the possibility of mimicking the in vivo situation of the osteoblasts. DeVore and North [15, 16] implanted acid-extracted, reconstituted calf skin collagen that had been cross-linked with aldehydes in rabbits and in guinea pigs and the graft encouraged healing by osteoconduction, providing a scaffold onto which bone cells could migrate. More recently, Deporter et al. demonstrated that fibrillar collagen enhances bone healing of calvarial defects [17].

Many researchers throughout the world are attempting to produce a functional, biological prosthetic graft for use in humans, even though the optimal concentration of collagen for coating has not been determined. The information obtained has been primarily descriptive and has provided limited insights into the factors responsible for the recruitment, attachment, and differentiated functions of cells interacting with the biomaterial implant. Cell culture models would offer the possibility of evaluating the effects of biomaterials on a variety of cellular and biochemical processes including cell attachment, morphology, cytoskeletal organization, proliferation, and expression of a differentiated phenotype.

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Therefore, in the present study we have assessed the potential of collagen type I both in bi-dimensional ("on gel" culture) and three-dimensional (spoon culture) structures to influence a variety of in vitro morphological and biochemical processes of a human osteoblastic cell line. Phenotypic differentiation was observed in vitro both in cells cultured on collagen-coated dishes and inside the collagen sponges.

Materials and Methods

Chemicals

Media and sera for cell cultures were purchased from Gibco (Grand Island, NY), and tissue culture plasticware was obtained from Falcon (Oxnard, CA). The Saos-2 cells, a clonal cell line derived from a human osteosarcoma, was obtained through the American Type Culture Collection (Rockville, MD). Synthetic 1-84 rat parathyroid hormone (rPTH(1-84)) and vasoactive intestinal peptide (VIP) were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Rhodamine phalloidin was obtained from Molecular Probes (Junction City, OR). The radioimmunoassay (RIA) kit for osteocalcin was purchased from Immunonuclear Corporation (Stillwater, MN). The Collagen CONDRESS was obtained from Gentili Institute (Pisa, Italy). Other reagents used were obtained at the purest grade commercially available.

Cell Cultures

The human osteosarcoma cell line, Saos-2, is used widely for studies on bone cell metabolism and differentiation. Saos-2 osteoblastic cells exhibit capability of synthesizing alkaline phosphatase and osteocalcin [18]. The cells show also a sensitive adenylate cyclase to PTH and VIP, lacking, however, any positive reaction for osteocalcin [18, 19]. Saos-2 cells were cultured in Coon's modified Ham's F12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 95% air-5% CO2 at 37°C. Detachment was achieved in 0.05% trypsin/1 mM EDTA for 3 minutes. Unless indicated, the experiments, including the preincubation procedures, were carried out in the steady-state medium (serum-free Coon's modified Ham's F12 medium). Cell viability was assessed by trypan blue dye exclusion.

Preparation of Collagen Substra

The Collagen CONDRESS was purified from bovine Achilles tendon after extraction with a nondenaturing procedure. The tendons were trimmed and extracted in 0.5 M acetic acid (5 liters/kg of organic material), at room temperature for 24-48 hours. The solution was passed through muslin to remove gross insoluble material. The collagenous material was then precipitated and aggregated into native bundles by raising ionic strength with NaCl at a final concentration of 2.5 M. The precipitate was then washed several times with distilled water and resuspended in 0.5 M acetic acid. The precipitation and successive dilution were repeated several times and then 100-200 ml of solution was dialyzed for periods of 24 hours against 0.5 M acetic acid. The final collagen solution contained 0.95 ± 0.02 mg/ml of collagen, as estimated from hydroxyproline [20]. These values were comparable to the bovine Achilles tendon standard offered by Calbiochem (La Jolla, CA). This solution was sterilized under gamma rays (0.5-1.5 Mrad) and kept indefinitely at 4°C Lyophilization was used for the preparation of the collagen sponges, where the fibrils maintained a banding pattern that could be observed by electron microscopy.

The collagen solution was used as such or diluted in 0.5 M acetic acid and dispersed in multiwell or Petri dishes (100 μl/sq cm) to provide a bi-dimensional substrate. After 24-hour incubation at 37°C, dishes were soaked five times with Coon's modified Ham's F12 medium. Non-specific binding sites were then blocked by incubating the wells with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 3 hours and by washing the dishes twice with PBS. Collagen substrata were then allowed to dry in tissue culture wells.

Both collagen-coated culture dishes and collagen sponges were sterilized overnight under the UV light before plating the Saos-2 cells.

In order to favor culture of Saos-2 cells inside the sponges, a cell suspension in steady state medium was injected with a large needle in the inner part of 10-mm collagen disks and allowed to settle for 24 hours before adding other medium.

Cell Attachment Assay

Saos-2 cells (5 × 104 cells/well) were added and incubated for the time indicated in 2-well Labtek tissue culture chamber/slides treated with different concentrations of collagen type I. Cells grown on plastic surfaces were used as controls. Incubations were performed in steady state medium. Saos-2 cells were also incubated 3 hours prior to harvesting in steady state medium containing 25 μg/ml cycloheximide, and the subsequent attachment assay was carried out in the presence of the same amounts of cycloheximide. The wells were washed twice with PBS, and adherent cells were fixed with 1% paraformaldehyde in PBS for 30 minutes, stained, and counted by a computerized digitizer at a magnification of 100X. Collagen was tested in quadruplicate in three separate experiments and expressed as mean ± SD percent of attached cells.

Light and Electron Microscopy

Adhesion and spreading of Saos-2 cells on top of collagen substrata was monitored with contrast phase and scanning electron microscopy (SEM).

For SEM, the specimens were fixed for 3–6 hours at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed for 1 hour at 4°C in 1% O3O2 in 0.1 M veronal acetate buffer (pH 7.4), dehydrated in graded ethanol-water solutions, critical-point-dried from CO2, sputter-coated with gold, and examined in a Philips 515 SEM at 15 kV accelerating voltage.

Saos-2 cells growing inside the collagen sponges were analyzed by transmission electron microscopy (TEM). The material for TEM was quickly fixed for 3–6 hours at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and postfixed for 1 hour at 4°C in 1% O3O2 in 0.1 M veronal acetate buffer (pH 7.4). The tissue blocks were stained en bloc by immersion in 2% uranyl acetate in pure alcohol, dehydration was done in graded ethanol-water solutions, diaphanization in propylene oxide, and embedding in epoxy resin. Polymerization was made in Beem 00 capsules overnight at 60°C in an embedding oven. Semithin sections, 0.5–1.0 μm thick, were cut from each block, stained with toluidine blue and cosin-erythrosin [21], and examined by light microscopy. For TEM, areas of interest were chosen after examining the semithin sections. After trimming the blocks, ultrathin serial sections were cut at 40–60 nm using a diamond knife, placed on formvar-coated Cu/Rh grids, stained with uranyl acetate and lead citrate, and examined in a Philips 410 LS TEM at 100 kV accelerating voltage.

Immunofluorescence for Actin Filaments

Cells grown on plastic and collagen-treated Labtek culture chamber/slides in growth medium were exposed to the steady state medium for 96 hours and fixed in freshly prepared 1% paraformaldehyde in PBS for 30 minutes at room temperature. Cells were permeabilized with 0.01% Nonidet P-40 in PBS for 30 minutes and rinsed twice with PBS. Rhodamine-conjugated phalloidin (1:20 dilution) was added to each well for 30 minutes. Before viewing each disk, a drop of 2.5% N-propyl gallate in 1:1 mixture of PBS and glycerol was added. The preparations were observed using a Leitz Dialux 20 microscope equipped with 100-W exciting source.

Cyclic AMP Accumulation

Saos-2 cells (5 × 104 cells/well) maintained in 24-well plates were