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OSTEOPONTIN EXPRESSION IN COCULTURE OF DIFFERENTIATING RAT FETAL SKELETAL FIBROBLASTS AND MYOBLASTS

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SUMMARY

Skeletal fibroblasts in vitro can acquire myofibroblast phenotypes by the development of biochemical and morphological features, mainly the expression of alpha–smooth-muscle actin (α-SMA). Myogenic differentiation is a central event in skeletal muscle development, and has commonly been studied in vitro in the context of skeletal muscle development and regeneration. Controlling this process is a complex set of interactions between myoblasts and the extracellular matrix. Osteopontin (OPN) is an acidic, phosphorylated matrix protein that contains an Arg-Gly-Asp (RGD) cell attachment sequence and has been identified as an adhesive and migratory substrate for several cell types. The aim of this study was to investigate osteopontin expression during the differentiation of skeletal fibroblasts into myofibroblasts and during myogenesis in a coculture model. Fibroblasts and myoblasts were obtained from skeletal muscle of 18-d-old Wistar strain rat fetuses by enzymatic dissociation. At 1 and 9 d, cocultures were immunolabeled, and the cells were also separately subjected to Western blotting to analyze OPN expression. Our data using confocal microscopy showed that myoblasts displayed a strong staining for OPN and that this labeling was maintained after myotube differentiation. Conversely, during fibroblast differentiation into myofibroblasts, we observed a significant increase in OPN expression. The results obtained by immunolabeling were confirmed by Western blotting. We suggest that OPN is important mainly during early stages of myogenesis, facilitating myoblast fusion and differentiation, and that the increased expression of OPN in myofibroblasts might be related to its effects as a key cytokine regulating tissue repair and inflammation.

Key words: skeletal fibroblast; skeletal myofibroblast; myogenesis; osteopontin.

It has been largely demonstrated that myofibroblasts play a key role in wound repair, inflammatory responses, growth, and development (Powell et al., 1999; Tomasek et al., 2002). We showed recently that fibroblasts obtained from skeletal muscle can acquire, in vitro, myofibroblast phenotypes, developing some biochemical and morphological features such as the expression of alpha–smooth-muscle actin (α-SMA) and increased number of lipid bodies, suggesting that after an injury, fibroblasts from the connective tissue surrounding the skeletal muscle might differentiate into myofibroblasts and could function as paracrine cells in the injured site (Pereira et al., 2004).

Cultures of skeletal myoblasts have been extensively used in the study of skeletal muscle development (Langen et al., 2003). Indeed, the irreversible transition from the proliferation-competent myoblast stage into fused, multinucleated myotubes, known as myogenic differentiation, has commonly been studied in vitro in the context of skeletal muscle development and regeneration (Yun and Wold, 1996). Controlling the onset and progression of this process is a complex set of interactions between myoblasts and their environment. Several lines of evidence demonstrate the importance of extracellular matrix molecules as part of the signaling mechanism in myogenesis (Melo et al., 1996).

Among the proteins present in the extracellular matrix, osteopontin (OPN), an acidic, phosphorylated glycoprotein that contains an Arg-Gly-Asp (RGD) cell attachment sequence (Oldberg et al., 1986), has been detected in several cell types, such as vascular smooth muscle cells (VSMC) (Liaw et al., 1994), endothelial cells, adventitial fibroblasts (Li et al., 2002), leukocytes (Bayless et al., 1997; Singh et al., 1999), macrophages (Murry et al., 1994), cardiomyocytes (Singh et al., 1999; Graf et al., 1997), skeletal muscle cells (Murry et al., 1994), and myofibroblasts (Hartner et al., 2001). This glycoprotein has been implicated in a variety of functions, depending on the cell type and on the stimuli that lead to its expression and secretion. For instance, it has been described as an adhesive and migratory substrate (Bayless et al., 1998; Komatsubara et al., 2003), a chemoattractant (Hirata et al., 2003), and a regulator of tissue repair and inflammation (O'Regan and Berman, 2000) in different tissues.

It is noteworthy that adhesive interactions are recognized requirements for cellular proliferation, migration, and differentiation during normal morphogenesis, as well as in diseases (Giachelli et al.,1995). OPN has been proposed to act as an adhesion molecule via its RGD

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motif and may play an important function in cell accumulation, migration, and proliferation, mainly within injured tissues (Ashizawa et al., 1996), showing an important role in the early inflammatory phase (Komatsubara et al., 2003). Specifically, it was demonstrated that OPN is highly up-regulated during muscle regeneration and may have an accessory role in this process (Hirata et al., 2003). In the present work, we analyzed OPN expression in a coculture of differentiating fetal skeletal myoblasts and fibroblasts, through immunofluorescence and Western blotting.

Cocultures of fibroblasts and myoblasts were obtained from thigh muscles of 18-d-old Wistar strain rat fetuses. Skeletal muscle was minced and enzymatically dissociated for 5 min with 0.05% trypsin and 0.01% versene in phosphate-buffered saline (PBS). After tissue dissociation, the cell suspension was centrifuged and the final pellet, containing fibroblasts and myoblasts, was resuspended in Dulbecco modified Eagle medium (DMEM) supplemented with 5% horse serum, 10% fetal calf serum, 2% chick embryo extract, and 50 μg/ml streptomycin. The cells (5.0×10^4 cells/well) were plated together in 24-well plates with glass coverslips precoated with 0.01% porcine gelatin, for immunofluorescence studies, and also separately in culture flasks, for Western blotting analysis. To separate fibroblasts from myoblasts, the cells were initially plated in flasks for 30 min to allow adhesion of fibroblasts, and then the supernatant containing the myoblasts was removed and plated into other flasks. The cells were then maintained at 37° C in a 5% CO_2 atmosphere in culture medium, for differentiation studies at 1 and 9 d. Twelve fetuses were used for each experiment, and an average of three wells of cells per animal. All the experiments were repeated at least three times.

To analyze the expression of OPN in differentiating fibroblasts and myoblasts simultaneously, cocultures at 1 and 9 d were fixed on coverslips with 4% paraformaldehyde in PBS and then were immunolabeled with a goat anti-osteopontin primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:100, using a rabbit biotinylated anti-goat secondary antibody and Cy-3 streptavidin to reveal the reaction. The cells were observed under an Olympus CLSM FV 300. Our results showed that, at 1 d of coculture (Fig. 1 *A–C*), OPN staining was positive in both fibroblasts and myoblasts; however, this labeling was more intense in myoblasts and was maintained after myotube differentiation (Fig. 1 *D–F*). We also observed that after the differentiation of fibroblasts into myofibroblasts there was an increase in OPN expression revealed by a strong positive staining scattered throughout the myofibroblast cytoplasm (Fig. 1 *D–F*). In addition, cocultures at 9 d showed that both myofibroblasts and myotubes were intensely labeled.

To show proper immunoreactive protein, and to assess the possible posttranslational condition of OPN in day 1 and day 9 cultures, Western blots were run on cell extracts. The same amount (5.0×10^6 cells) of fibroblasts, myofibroblasts, myoblasts, and myotubes that were plated separately in culture flasks, were lysed in 200 μl of HES buffer (0.3 m*M* *N*-2-hydroethylpiperazine-*N*-2-ethane-sulfonic acid('), 5 m*M* ethylenediamine-tetraacetic acid, 0.1 m*M* sodium orthovanate, 10 μl/ml Triton X-100, 0.1 *M* sodium fluorite, 0.1 *M* sodium pyrophosphate, 10μg/ml aprotinin, and 10μg/ml leupeptinin) for 10 min. The cell extract was then centrifuged for 20 s at $42{,}000 \times g$ at 4° C and the total protein content present in the supernatant was determined by the Bradford method. Cellular proteins (40 μg total) were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, and were transferred to a polyvinylidene difluoride filters. The primary antibody used was a goat anti-osteopontin (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The Polyvinylidene difluoride (PVDF) filters were next incubated with appropriate secondary antibody conjugated to biotin followed by 1 h incubation with horseradish peroxidase-conjugated streptavidin. Immunoreactive proteins were visualized by 3,3′-diaminobenzidine staining, and the bands were quantified by densitometry, using Image J Software (National Institutes of Health, Bethesda, MD).

Western blotting data (Fig. 2) showed that, when analyzed separately, the cells displayed the same profile of OPN expression as was observed through immunofluorescence. We demonstrated that at 9 d, after the differentiation of fibroblasts into myofibroblasts, there was a statistically significant increase in OPN expression. However, during myogenesis, there was no difference in OPN expression when we compared myoblasts at 1d to myotubes at 9 d. The results were expressed as mean ± standard error of mean and were statistically analyzed by Student's *t*-test, using a significance level of $P < 0.05$.

Cultures of skeletal myoblasts have been extensively used in the study of skeletal muscle development and regeneration (Langen et al., 2003). It is known that in muscle injuries the release of growth factors is an important step in the initiation of the healing process, stimulating the growth and the differentiation of various muscle-derived cells (Li and Huard, 2002). Recently, it was shown that OPN is up-regulated during the skeletal muscle regeneration process (Hirata et al., 2003). In the present study, we analyzed OPN expression during the differentiation of skeletal fibroblasts into myofibroblasts and during the fusion of myoblasts into myotubes in a coculture model.

OPN, a matricellular glycoprotein, is found in a large variety of tissues, and has been implicated in different functions, including cell adhesion, migration, proliferation, and differentiation. Several lines of evidence demonstrate the importance of extracellular matrix molecules as part of the signaling mechanism in myogenesis and muscle regeneration (Buck and Horwitz, 1987; Lafuste et al., 2005). It has been shown that the binding of RGDS (Arg-Gly-Asp-Ser) peptides or antibodies to integrin receptors in myoblast cultures inhibits cell fusion and further differentiation (Boettiger et al., 1995; Melo et al.,1996; Lafuste et al., 2005). In this study, we observed high expression of OPN during myogenesis in both myoblasts at 1 d and multinucleated myotubes at 9 d. We suggest that this early expression could be related to a specific role played by OPN during myoblast proliferation, migration, and fusion. Because OPN has a RGD sequence, it could function as an adhesive protein, binding myoblasts to other matrix proteins, facilitating myogenesis.

It is noteworthy that OPN has been described as a key cytokine regulating tissue repair and inflammation (O'Regan and Berman, 2000). Interestingly, it has been also demonstrated that myofibroblasts have an important function in tissue repair and inflammatory responses (Tomasek et al., 2002). Recently, we have demonstrated that skeletal fibroblasts can differentiate into myofibroblasts in vitro, acquiring some phenotypic characteristics such as α-SMA expression and an increased number of lipid bodies in their cytoplasms (Pereira et al., 2004). In the present work, we observed that skeletal fibroblasts at 1 d expressed OPN, and during their differentiation into myofibroblasts, they exhibited an accentuated increase in this expression.

It was demonstrated that up-regulation of the OPN gene and of

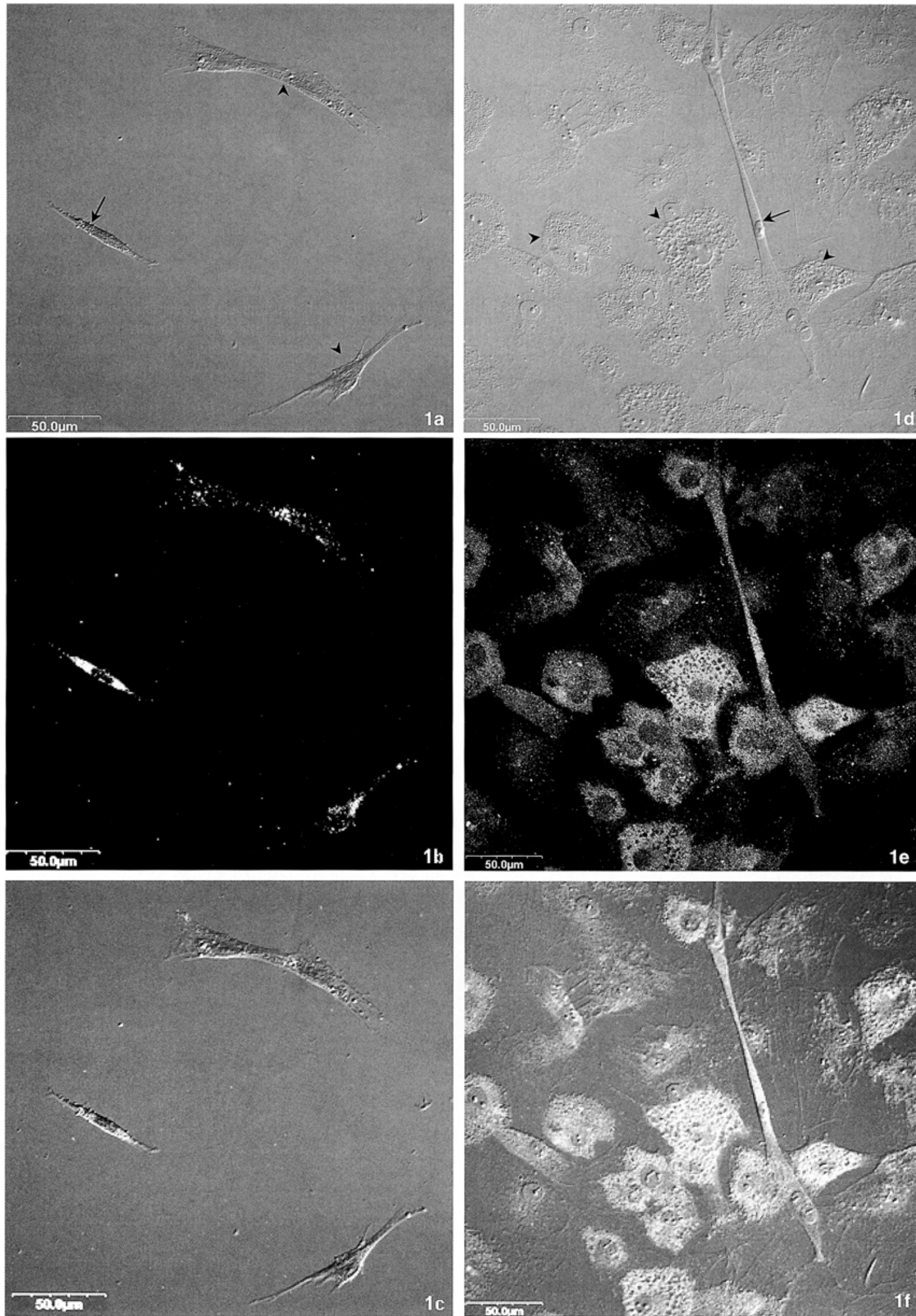

FIG. 1. Confocal microscopy of cocultures immunolabeled at 1 d (left column, ×40) and 9 d (right column, ×20). Myoblasts (arrow) and fibroblasts (arrowhead) at 1 d, and myotubes (arrow) and myofibroblasts (arrowhead) at 9 d were observed using a differential interference contrast filter (1*A* and 1*D*), fluorescence filter (1*B* and 1*E*) and merging of differential interference contrast and fluorescence (1*C* and 1*F*). Bar = 50µm.

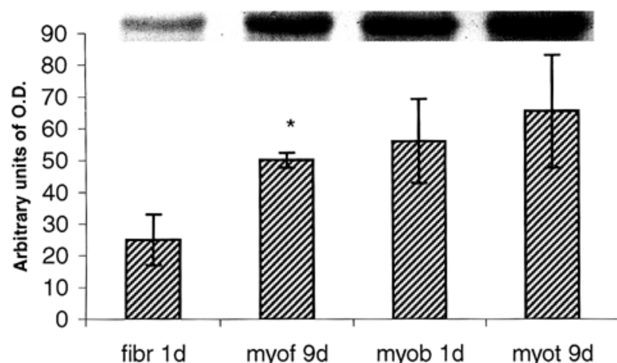

FIG. 2. Representative Western immunoblot of OPN (50 kDa) in cultures of fibroblasts (fibr) and myoblasts (myob) at 1 d, and myofibroblasts (myof) and myotubes (myot) at 9 d. Analysis of proteins was performed by optical densitometry and is summarized in this bar graph. Results are expressed in arbitrary densitometry units (n = 6 group; $*P < 0.05$).

several other unknown genes may be involved in the phenotypic transition of adventitial fibroblasts to myofibroblasts, and might play an important role in vascular remodeling (Sun et al., 2001). Moreover, in an experimental model of chronic glomerulosclerosis, glomerular OPN mRNA and protein were detected in activated myofibroblasts (Hartner et al., 2001). Because fibroblast differentiation into myofibroblasts can be triggered after a tissue injury, we suggest that the increased expression of OPN in myofibroblasts might be related to its role in wound healing and inflammatory responses. Therefore, OPN could have an effect on fibroblast differentiation and a role in myofibroblast function during tissue remodeling.

Taken together, our results show that OPN has a specific expression pattern in each cell type analyzed during differentiation within our coculture system. We hypothesize that this specific expression pattern might be related to adhesive and regulatory functions of OPN during muscle-derived cell differentiation, and probably also during muscle regeneration.

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