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# Shark Cartilage Extract Interferes with Cell Adhesion and Induces Reorganization of Focal Adhesions in Cultured Endothelial Cells

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**Abstract** In this study, we examined the effects of shark cartilage extract on the attachment and spreading properties and the focal adhesion structure of cultured bovine pulmonary artery endothelial cells. Treatment with cartilage extract resulted in cell detachment from the substratum. Immunofluorescence staining of those treated cells that remained attached showed that, instead of being present in both central and peripheral focal adhesions as in control cells, both integrin $\alpha_v\beta_3$ and vinculin were found only in peripheral focal adhesion and thinner actin filament bundles were seen. In addition to causing cell detachment, cartilage extract partially inhibited the initial adherence of the cells to the substratum in a dose-dependent manner. Integrin $\alpha_v\beta_3$ and vinculin staining of these cells also showed a peripheral focal adhesion distribution pattern. Vitronectin induced cell spreading in the absence of serum, but was blocked by simultaneous incubation with cartilage extract, which was shown to inhibit both integrin $\alpha_v\beta_3$ and vinculin recruitment to focal adhesion and the formation of stress fibers. Dot binding assays showed that these inhibitory effects on cell attachment and spreading were not due to direct binding of cartilage extract components to integrin $\alpha_v\beta_3$ or vitronectin. Shark cartilage chondroitin sulfate had no inhibitory effect on either cell attachment or spreading of endothelial cells. These results show that the inhibitory effects of cartilage extract on cell attachment and spreading are mediated by modification of the organization of focal adhesion proteins. J. Cell. Biochem. 78: 417–428, 2000. 

**Key words:** shark cartilage extract; endothelial cells; focal adhesion; immunofluorescence

Cell-substratum adhesion is a critical step during cell growth, development, differentiation, and cancer metastasis [for review see Hynes, 1992]. The adhesion process is mediated by the formation of focal adhesions (FAs), which involves the binding of cell surface receptors, the integrins, to the extracellular matrix (ECM) via their extracellular domains. The intracellular domains of the integrins interact with α-actinin and talin, which, in turn, interact with actin and vinculin. In addition, several other proteins, such as focal adhesion kinase (FAK), tensin, paxillin, and protein kinase C (PKC), are also found in FAs [Lo and Chen, 1994; Yamada and Miyamoto, 1995; Craig and Johnson, 1996].

The organization of the FA is regulated by several factors [Schoenwaelder and Burrige, 1999]. Increased tyrosine phosphorylation of FAK, a protein tyrosine kinase, is accompanied by cell attachment to the ECM [Burridge et al., 1992] and FA assembly [Defilippi et al., 1995], while inhibition of tyrosine phosphatase activity leads to stress fiber and FA formation [Defilippi et al., 1995; Retta et al., 1996]. Treatment of serum-starved 3T3 fibroblasts with phenylarsine oxide, a tyrosine phosphatase inhibitor, results in increased tyrosine phosphorylation of FAK and paxillin [Retta et al., 1996],

Abbreviations used: CPAE, pulmonary artery endothelium; ECM, extracellular matrix; FA, focal adhesion; FAK, focal adhesion kinase; MEM, minimum essential medium; PKC, protein kinase C; SCE, shark cartilage extract.

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and the serum- or lysophosphatidic acid-induced reappearance of FAs and stress fibers in quiescent 3T3 fibroblasts is accompanied by increased tyrosine phosphorylation of FAK and other tyrosine kinases [Chrzanowska-Wodnicka and Burridge, 1994]. Tyrosine phosphatase activity is markedly increased during actin depolymerization and FA disassembly, which are blocked by phenylarsine oxide [Retta et al., 1996]. Furthermore, tyrosine phosphatase activation has been demonstrated during reduction of cell-substratum adhesion in chicken embryo fibroblasts [Maher, 1993].

In addition to tyrosine kinases and phosphatases, PKC is also found in FAs [Jaken et al., 1989]. PKCϵ translocates from the cytosol to the cell membrane when HeLa cells adhere to gelatin [Chun et al., 1996], and the translocation of PKCα and PKCϵ to FAs mediates smooth muscle cell spreading [Haller et al., 1998]. Moreover, activation of PKC precedes integrin-mediated cell spreading on fibronectin [Vuori and Ruoslahti, 1993]. Integrin $\beta_1$, talin, and vinculin are all colocalized in FAs [Woods and Couchman, 1992], and stress fiber assembly is induced by activation of PKC [Lewis et al., 1996]. It is clear that PKC plays a crucial role in cell adhesion and spreading, as well as in FA formation [Greenwood and Murphy-Ullrich, 1998].

Cell adhesion can also be affected by modification of ECM components. Proteoglycans from nasal and articular hyaline cartilage [Knox and Wells, 1979; Rich et al., 1981; Yang et al., 1998; Noyori and Jasin, 1994], chick embryo fibroblasts [Yamagata et al., 1989], and rat tumor cells [Brennan et al., 1983] have been shown to inhibit cell adhesion to extracellular substrates, including collagen and fibronectin. In contrast, perlecan, a heparan sulfate proteoglycan and a component of cartilage matrix, is able to promote chondrocyte attachment [SundarRaj et al., 1995]. The mechanism of inhibition of cell adhesion varies as a function of the properties of the proteoglycans, which may interact with the cell surface [Knox and Wells, 1979], the substratum [Rich et al., 1981; Brennan et al., 1983; Bidanset et al., 1992; Noyori and Jasin, 1994], or neither [Yamagata et al., 1989]. The signaling pathway responsible for the decreased cell adhesiveness to the substratum following proteoglycan treatment is unknown.

In contrast to the inhibitory effect of proteoglycans on cell adhesion, thrombospondin enhances bovine aortic endothelial cell adhesion [Murphy-Ullrich and Höök, 1989; Greenwood et al., 1998]. Although thrombospondin promotes cell adhesion, FAs, and stress fibers undergo disassembly and vinculin and stress fibers are found only in the peripheral cytoplasm when cells attach to thrombospondin [Murphy-Ullrich and Höök, 1989]. Similar results were obtained when Chinese hamster ovary cells were allowed to attach to intact fibronectin preincubated with dermatan sulfate proteoglycan [Bidanset et al., 1992]. Recent results by Greenwood et al. [1998] demonstrate that activation of phosphoinositide 3-kinase is responsible for thrombospondin-induced FA disassembly.

Although cartilage from various sources has been used to study cell adhesion and although shark cartilage has been shown to inhibit angiogenesis [Sheu et al., 1998; Davis et al., 1997; Oikawa et al., 1990; Lee and Langer, 1983], the effect of shark cartilage on cell adhesion has not yet been reported. In addition, shark cartilage-induced morphological changes in FA proteins have not been studied. In this report, we examined the effect of shark cartilage extract (SCE) on the adhesive properties of bovine pulmonary artery endothelial (CPAE) cells. Since the cell adhesion process can be divided into cell attachment and spreading, FA formation, and stress fiber assembly [Greenwood and Murphy-Ullrich, 1998], we focused primarily on the effect of SCE on FA organization in an attempt to correlate cell adhesion with FA structure.

## MATERIALS AND METHODS

### Cell Culture

Pulmonary artery endothelial (CPAE) cells (CCL-209), established from the main stem pulmonary artery of a young cow (Bos taurus), were purchased from the American Type Culture Collection (Rockville, MD) and cultured in minimal essential medium (MEM; Gibco, Long Island, NY), supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. In this study, the passage number of CPAE cells was between 17 and 23.

### Preparation of SCE

Shark cartilage powder was extracted with 1 M guanidine HCl and the solubilized mate-

rial precipitated with alcohol. Each milligram wet weight of the precipitated material contained 6 μg of proteins and 18.9 μg of carbohydrates. Before use, the precipitated material was resuspended in serum-free minimal essential medium and the protein concentration determined using the Bio-Rad protein assay kit (Richmond, CA).

### Cell Adhesion and Spreading Assay

For the adhesion assay, CPAE cells, prepared by detaching cells from the substratum by trypsinization, were transferred to 96-well plates and allowed to attach in the presence of SCE, purified shark cartilage chondroitin sulfate (Sigma, St. Louis, MO), or control medium. Cell numbers in each well were estimated using the acid phosphatase assay, the absorbance of the solution being proportional to the number of cells (see below). For immunofluorescence studies, trypsinized cells were plated on uncoated cover-glasses in the presence or absence of SCE.

For the cell spreading assay, CPAE cells were first allowed to attach to uncoated coverglasses for 30 min, then induced to spread by various agents. The cells were then either photographed on an Olympus inverted microscope or fixed with formalin for immunofluorescence staining.

### Acid Phosphatase Assay

The acid phosphatase assay was performed as described by Connolly et al. [1986]. Briefly, the medium was aspirated and the cells in each well washed once with 200 μl of PBS before being incubated for 2 h at 37°C with 100 μl of 0.1 M sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 and 10 mM p-nitrophenyl phosphate (Sigma 104 phosphatase substrate). The reaction was stopped by the addition of 10 μl of 1 N sodium hydroxide and the absorbance of the solution read at 405 nm using an ELISA reader (Bio-Tek Instruments, Winooski, VT). The first well of each plate, without any cells, was used as a control. A linear correlation was obtained between enzyme activity and cell number over the range of 100–10,000 cells per well. The results for the different treatment groups were expressed as the mean ± SD.

### Immunofluorescence Microscopy

Cells cultured on coverslips were fixed for 10 min at room temperature with 10% formalin, then permeabilized for 10 min at room temperature with 0.1% Triton X-100. After blocking for 30 min at room temperature with 5% non-fat milk in PBS, the cells were incubated at 37°C for 1 h with primary antibodies; the antibodies used were mouse monoclonal antibody against vinculin (Sigma), and mouse monoclonal antibodies against integrin $\alpha_v\beta_3$ (clone LM 609) and integrin β1, polyclonal rabbit anti-integrin α3 antibody, and rat monoclonal anti-integrin α6 antibodies (all from Chemicon, Temecula, CA). After PBS washes, the cells were reacted for 1 h at 37°C with Texas red-conjugated anti-mouse, rabbit or rat IgG (Vector Laboratory, Burlingame, CA), followed by FITC-conjugated phalloidin (Sigma) for 20 min, briefly washed, and mounted. The immunofluorescence images were photographed using a Zeiss epifluorescence microscope (Zeiss, Inc., Thornwood, NY).

### Dot Immunoassay

Nitrocellulose membrane strips were briefly rinsed with Tris base saline (TBS; 150 mM NaCl, 50 mM Tris base, pH 8.2), then air-dried. SCE (100 μg/ml), integrin $\alpha_v\beta_3$ (1 μg/ml; Chemicon), and vitronectin (25 μg/ml; Sigma) were then dotted onto separate membrane strips. To test the binding of SCE to integrin $\alpha_v\beta_3$ or vitronectin, the SCE-dotted membrane strip was incubated with integrin $\alpha_v\beta_3$ or vitronectin, then with mouse anti-integrin $\alpha_v\beta_3$ or anti-vitronectin antibody. As a positive control, integrin $\alpha_v\beta_3$- and vitronectin-dotted membrane strips were directly reacted with primary antibodies. After washing with TBS containing 0.1% Tween 20 to remove unbound antibodies, the membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Promega, Madison, WI), washed, and finally developed with a combination of nitro blue tetrazolium (NBT, Sigma) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Sigma) in alkaline phosphatase assay buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris base, pH 9.5).

## RESULTS

### SCE Induces Rearrangement of FA Proteins

In addition to inhibiting cell proliferation (data not shown), SCE also induced cell detachment, with about 50–60% of the treated cells becoming rounded-up and subsequently, de-

taching from the substratum. Since cells adhere to the extracellular substratum by FAs, we were therefore interested in knowing whether SCE modifies the structure of the FA. The integrin subtypes expressed in CPAE cells were first determined by immunofluorescence staining with antibodies specific for integrin $\alpha_3$, $\alpha_6$, $\beta_1$, or $\alpha_v\beta_3$. Since the only antibody to give positive staining of FAs was the anti-$\alpha_v\beta_3$ antibody, this was used throughout the study to viualize integrin distribution in the cells. Immunostaining of control CPAE cells with anti-integrin $\alpha_v\beta_3$ (Fig. 1A) and anti-vinculin (Fig. 1C) antibodies revealed typical thread-like FA patterns distributed throughout the cytoplasm, accompanied by abundant stress fibers (Fig. 1B,D). After SCE treatment, the remaining attached cells showed decreased staining for integrin $\alpha_v\beta_3$ (Fig. 1E) and vinculin (Fig. 1G) and both were preferentially lost from the central cytoplasm, becoming concentrated at the cell periphery. Phalloidin-FITC staining of the same cells showed that the typical long straight stress fibers crossing the cells were no longer present; instead, the actin filaments became thinner and more irregularly organized, with many dot-like structures (Fig. 1F,H, arrowheads) along the length of the bundles, forming numerous polygonal networks. Furthermore, integrin $\alpha_v\beta_3$, vinculin, and actin staining of the detached cells was completely diffuse. These results indicate that SCE induces cell detachment by modification of the distribution and structural integrity of some FA proteins.

### Effect of SCE on Cell Adhesion During Plating

We then studied the effect of SCE on cell adhesion. When SCE was added for 1 h to the plating medium, cell adhesion was inhibited in a dose-dependent manner (Fig. 2); this occurred even in the presence of serum, but was even more pronounced in serum-free medium. After removal of SCE, the attached rounded-up cells assumed a normal morphology after 2 h and started to proliferate within 24 h; immunofluorescence studies of these attached cells revealed that polymerization of actin filaments was not affected, but that, even after 3 h, the thin actin bundles were not able to organize into typical stress fibers (Fig. 3B,D), whereas control cells adhered in serum-containing medium displayed organized stress fibers (not shown). In addition, anti-integrin $\alpha_v\beta_3$ (Fig. 3A) and anti-vinculin (Fig. 3C) staining was mainly located at the cell periphery, as was the case in those cells that remained adherent following SCE treatment (Fig. 1E,G). SCE-induced inhibition of cell attachment and changes in the organization of FA proteins were also seen when SCE was first immobilized on the cover-glass (data not shown). These experiments show that SCE not only modified preformed FAs, but also retarded the normal organization of FA proteins and the formation of stress fibers during cell adherence.

### SCE Inhibits Vitronectin-Induced Cell Spreading

We then examined the effect of SCE on cell spreading. Previous studies [Vuori and Couchman, 1992; Chun et al., 1996; Haller et al., 1998] on cell adhesion and spreading using ECM component-coated slides did not distinguish between cell attachment and spreading, since the cells simultaneously attached to the coated slides and spread. In order to study cell attachment and spreading separately, CPAE cells were first allowed to adhere onto uncoated cover-glasses for 30 min in the absence of serum. Figure 4A shows that, under these condition, the cells remained rounded-up. Addition of serum (Fig. 4B) or vitronectin (5 μg/ml; Fig. 4C) induced the formation of large lamellipodia within 1 h. While vitronectin-induced cell spreading was inhibited by SCE (300 μg/ml; Fig. 4D), serum-mediated spreading was not affected by SCE (Fig. 4E). Immunofluorescent studies of rounded CPAE cells plated in the absence of serum showed that staining for integrin $\alpha_v\beta_3$ (Fig. 5A), vinculin (Fig. 5B), and phalloidin was diffusely distributed throughout the cell and that no typical thread-like FAs and stress fibers were present. Soluble vitronectin induced the recruitment of integrin $\alpha_v\beta_3$ (Fig. 5D) and vinculin to FAs after 1 h of treatment (Fig. 5E) and typical FAs were seen at the periphery, while those in the central cytoplasm appeared finer and shorter. The associated actin filaments were initially loosely arranged (Fig. 5F), then later (at about 2 h) became organized into compact stress fibers, accompanied by distribution of integrin $\alpha_v\beta_3$ and vinculin throughout the cell (not shown). In contrast, the anti-integrin $\alpha_v\beta_3$ (Fig. 5G), anti-vinculin (Fig. 5H), and phalloidin (Fig. 5I) staining patterns of cells treated with a combination of SCE and vitronectin were the same as those seen in serum-free MEM. This result indicates

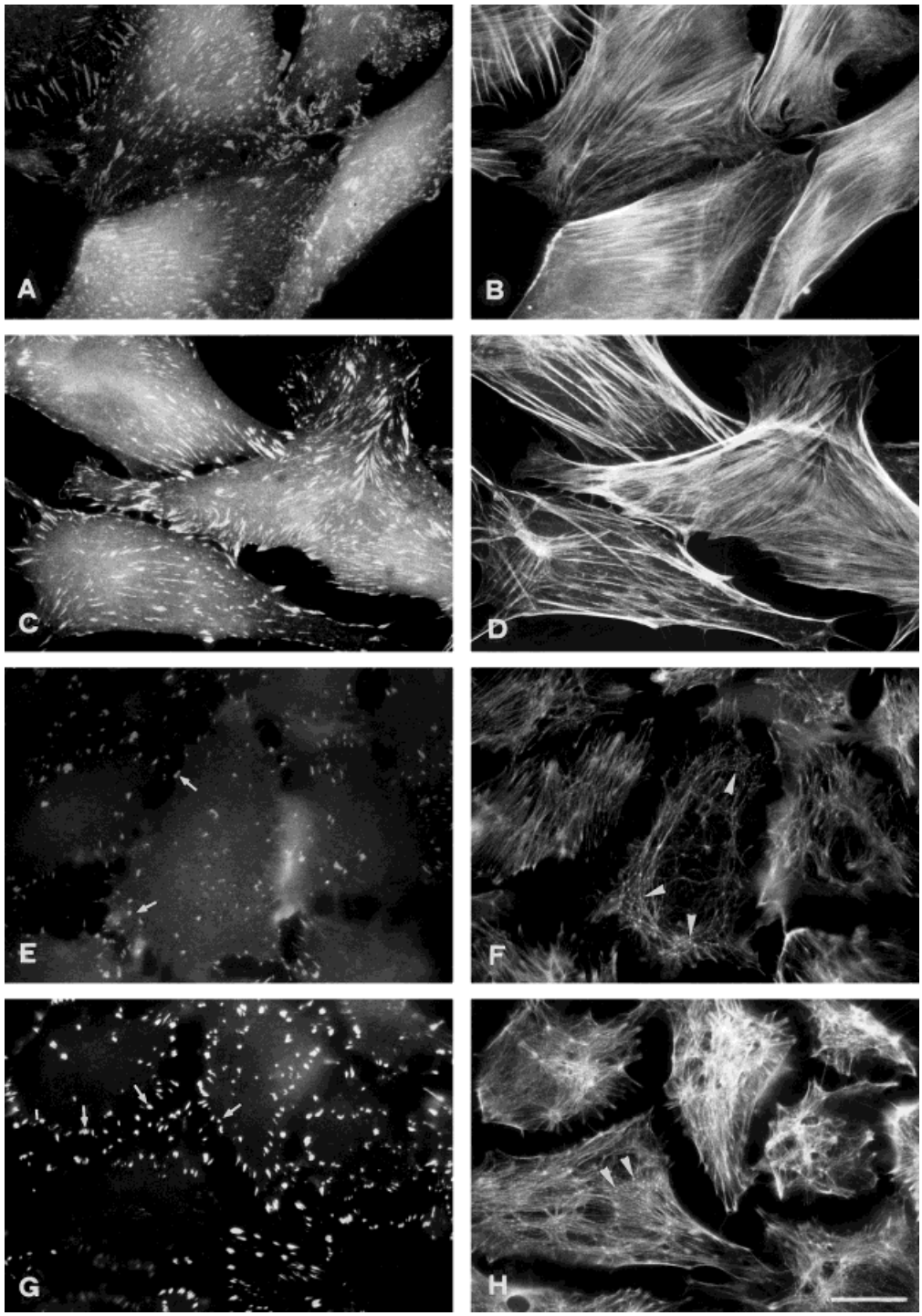


**Fig. 1.** Effect of SCE treatment on the distribution of focal adhesion proteins in CPAE cells. Control (A–D) and SCE-treated (E–H) cells were stained with anti-integrin $\alpha_v\beta_3$ (**A,E**) or anti-vinculin (**C,G**) antibodies, then with phalloidin-FITC (**B,D,F,H**). Normal CPAE cells display many integrin $\alpha_v\beta_3$- (A) and vinculin- (C) containing focal adhesions and typical stress fibers in the cytoplasm. Following treatment with SCE (60 μg/ml) for 24 h, most of the integrin $\alpha_v\beta_3$ (arrows in E) and vinculin (arrows in G) staining is located in the peripheral cytoplasm, while that in the central cytoplasm is diffuse or absent. Compact stress fibers are replaced by thin actin bundles with many bright focal spots (arrowheads in F,H). In addition, adjacent actin bundles are connected in a polygonal network. Scale bar = 20 μm.

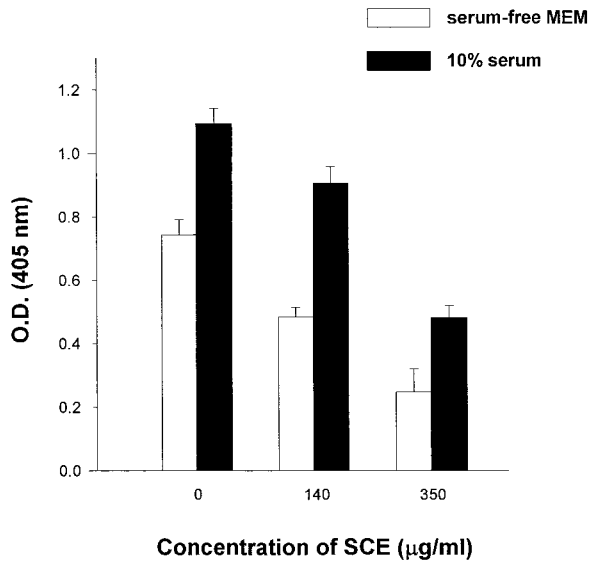


**Fig. 2.** Effect of SCE on cell adhesion. CPAE cells were plated for 1 h at 37°C in serum-containing (filled column) or serum-free (empty column) medium containing SCE (140 μg/ml or 350 μg/ml), then assayed for acid phosphatase activity.

that SCE may inhibit cell spreading by preventing the formation of FAs and stress fibers.

### SCE Does Not Bind Directly to Integrin $\alpha_v\beta_3$ or Vitronectin

Using a dot binding assay, we then tested whether the SCE-induced inhibition of cell adhesion and spreading was mediated through the binding of cartilage extract components to integrin or vitronectin. Neither integrin $\alpha_v\beta_3$ (Fig. 6C) nor vintronectin (Fig. 6F) bound to the immobilized SCE, as shown by the lack of binding of anti-integrin $\alpha_v\beta_3$ or anti-vitronectin antibodies, which in positive controls, respectively, recognized integrin $\alpha_v\beta_3$ (Fig. 6A) or vitronectin (Fig. 6D). Negative controls (Figs. 6B,E) showed background staining.

### Chondroitin Sulfate From Shark Cartilage Does not Inhibit Cell Adhesion

We then examined whether chondroitin sulfate, a major component of shark cartilage, was responsible for the inhibitory action of SCE on cell adhesion and spreading. Chondroitin sulfate (1 mg/ml) did not inhibit cell adhesion in the presence of serum (Fig. 7, FBS + CS group); in fact, it partially promoted cell attachment in serum-free MEM (Fig. 7, SF + CS group).

## DISCUSSION

Our results show that SCE interfered with cell adhesion to the culture substratum by modifying the organization of FAs and preventing the formation of stress fibers. SCE also affected FA structure and stress fibers in adherent cells, and this may be responsible for the subsequent detachment of the cells from the substratum. We also showed that these SCE-induced effects were not due to the presence of chondroitin sulfate in the SCE or the binding of SCE to integrin or vitronectin. Although integrin $\alpha_v\beta_3$ showed a marked redistribution following SCE treatment, we cannot exclude the possibility that other integrin subtypes were also expressed and reorganized by SCE treatment, but currently, there are no antibodies available for us to test this possibility.

The observation that, in those SCE-treated cells that remained attached, FA proteins were lost from the center of the cell, but remained at the periphery, suggests that SCE affects cell adhesion by modifying FA organization via a reduced distribution of integrin $\alpha_v\beta_3$ and vinculin. Moreover, cell-substratum adhesion was partially inhibited by SCE, the attached cells displaying only peripheral integrin and vinculin staining and lacking the organization of actin filaments into stress fibers. In addition, in cells in which spreading was induced by vitronectin, prominent FAs were seen at the cell edge, while those in the central region appeared immature. Taken together, these results indicate that the presence of peripheral FAs is sufficient for cells to attach to the substratum and spread. A previous study also showed that, during the attachment of endothelial cells to vitronectin, FAs were first formed at the cell edge, then subsequently appeared in the central cytoplasm [Dejana et al., 1988]. The reorganization of preformed FAs and the modification of FA assembly induced by SCE during cell adhesion are similar to those seen in cells attached to a substratum coated with either thrombospondin [Murphy-Ullrich and Höök, 1989] or dermatan sulfate proteoglycans [Bidanset et al., 1992], both of which act as extracellular substrates to support cell attachment, but result in disassembly of FA and stress fibers. In contrast, our results show that SCE not only affects cell attachment, but also inhibits cell spreading. It is well established that the effect of thrombospondin on FA

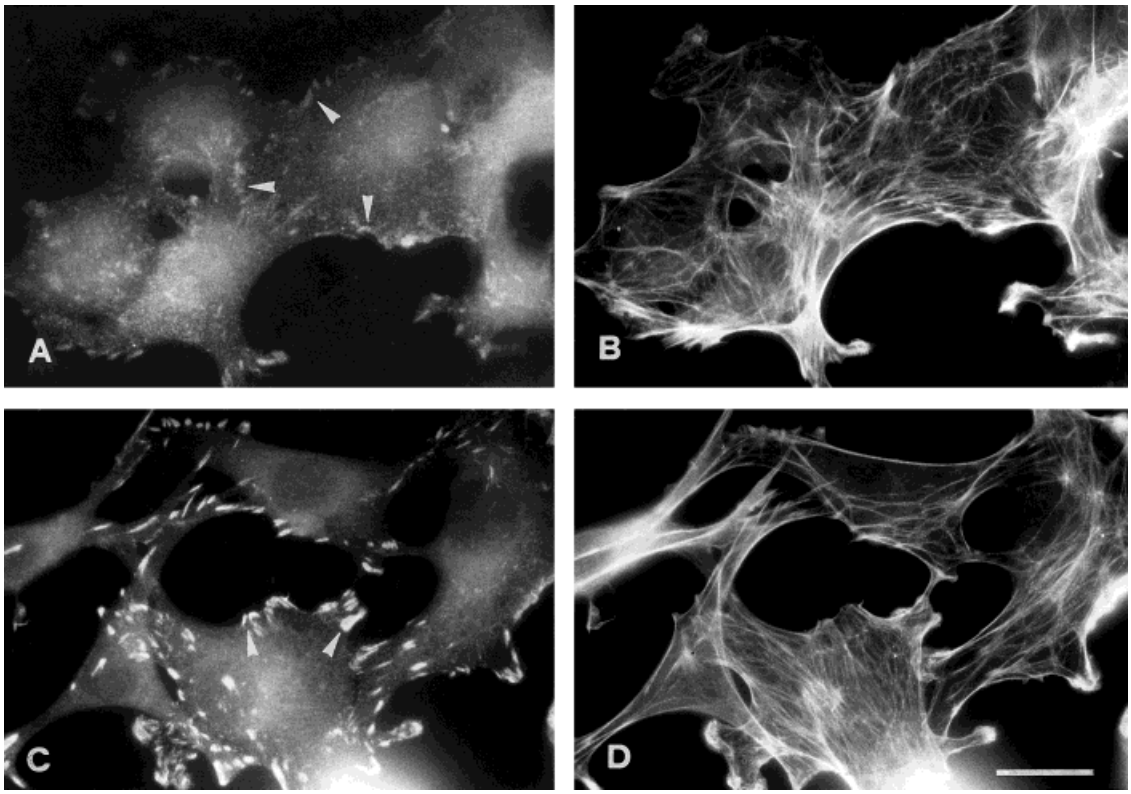


**Fig. 3.** Effect of SCE on the formation of focal adhesions and stress fibers during initial plating. CPAE cells were trypsinized and plated for 3 h in culture medium containing SCE (300 μg/ml) and serum, then double-labeled for F-actin (**B,D**) and integrin $\alpha_v\beta_3$ (**A**) or vinculin (**C**). Integrin $\alpha_v\beta_3$ (arrowheads in A) and vinculin (arrowheads in C) staining is distributed in the peripheral cytoplasm, and phalloidlin-FITC (B,D) staining reveals less organized thin actin bundles, but no authentic stress fibers. Scale bar = 20 μm.

disassembly is mediated via activation of phosphoinositide 3-kinase [Greenwood et al., 1998]; however, the mechanism involved in the SCE-induced cell detachment and modification of FA and stress fibers is still unknown.

Previous studies using a fibronectin-coated substratum could not differentiate between the two separate processes of cell attachment and spreading, since the attaching cells simultaneously started to spread. This problem was solved by first plating CPAE cells in serum-free MEM, conditions under which they remain rounded-up for at least 1 h; when vitronectin is subsequently added, cell spreading occurs, concomitantly with the formation of FA and stress fibers. This result clearly demonstrates that soluble vitronectin is also able to induce substratum-attached cells to spread, and that vitronectin, the major cell spreading factor in fetal bovine serum [Hayman et al., 1985], is necessary and sufficient for cell spreading and FA formation. The vitronectin-induced formation of FAs and stress fibers may involve a pathway different from that involving activation of Rho (see below), which requires lysophosphatidic acid, serum, or other growth factors to promote stress fiber formation.

Integrin-mediated adhesion triggers a series of events, of which tyrosine phosphorylation and PKC translocation have been shown to play important roles in the regulation of FA formation and stress fiber organization. Increased tyrosine phosphorylation of FAK [Short et al., 1998; Burridge et al., 1992; Schaller et al., 1992] is seen following cell adherence to fibronectin, and treatment with phosphotyrosine phosphatase inhibitors upregulates tyrosine phosphorylation of FAK and paxillin, resulting in FA and stress fiber formation [Defilippi et al., 1995; Retta et al., 1996]. Barry and Critchlay [1994] further demonstrated that vanadyl hydroperoxide, a tyrosine phosphatase inhibitor, induces the formation of stress fibers in quiescent Swiss 3T3 fibro-

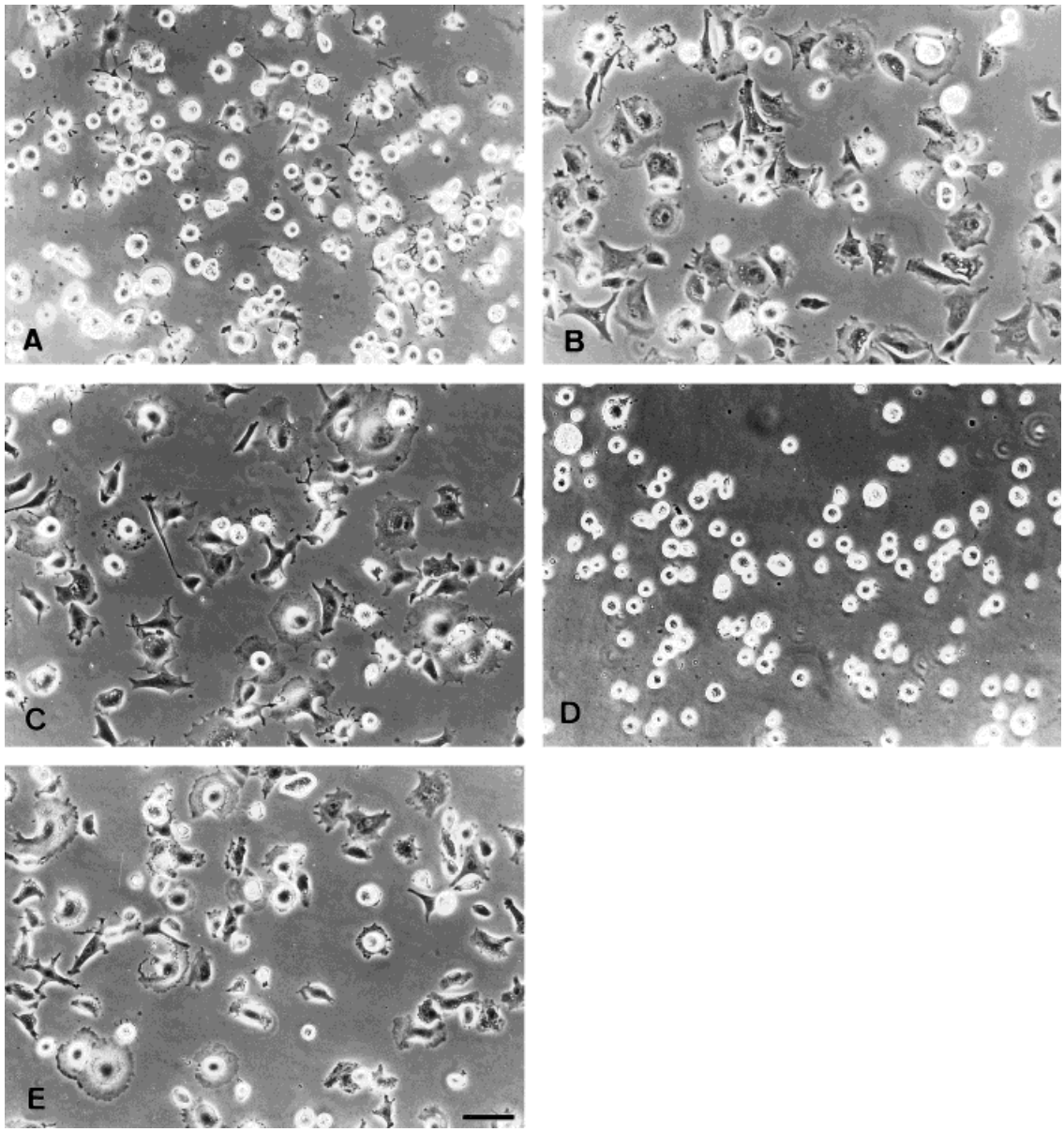


**Fig. 4.** Inhibition of cell spreading by SCE. CPAE cells were allowed to attach onto uncoated cover-glasses for 30 min and were then incubated under different conditions. **A:** Control cells: 1 h in serum-free MEM. Serum (**B**) or vitronectin (**C**) induces cell spreading with the formation of large lamellipodia within 30 min of treatment. Addition of SCE (300 μg/ml) inhibits vitronectin-induced spreading (**D**). In contrast, spreading of SCE-treated cells can be rescued by serum (**E**). Scale bar = 100 μm.

blasts, which lack stress fibers and FAs. Moreover, both reduction of cell-substrate adhesion [Maher, 1993] and cell detachment from the substratum [Retta et al., 1996] result in increased tyrosine phosphatase activity. It is therefore apparent that the balance between tyrosine kinase and phosphatase activity is critical for cell-substratum adhesion. Consistent with this notion is the finding that the anti-phosphotyrosine staining in SCE-treated CPAE cells was limited to the cell periphery and was lost when the cells became detached (not shown), suggesting the involvement of phosphotyrosine proteins. The role of FAK in cell adhesion is paradoxical, since Wilson et al. [1995] found that, in mouse aortic smooth muscle cells, adherence on fibronectin induces the formation of stress fibers and FAs without ac-

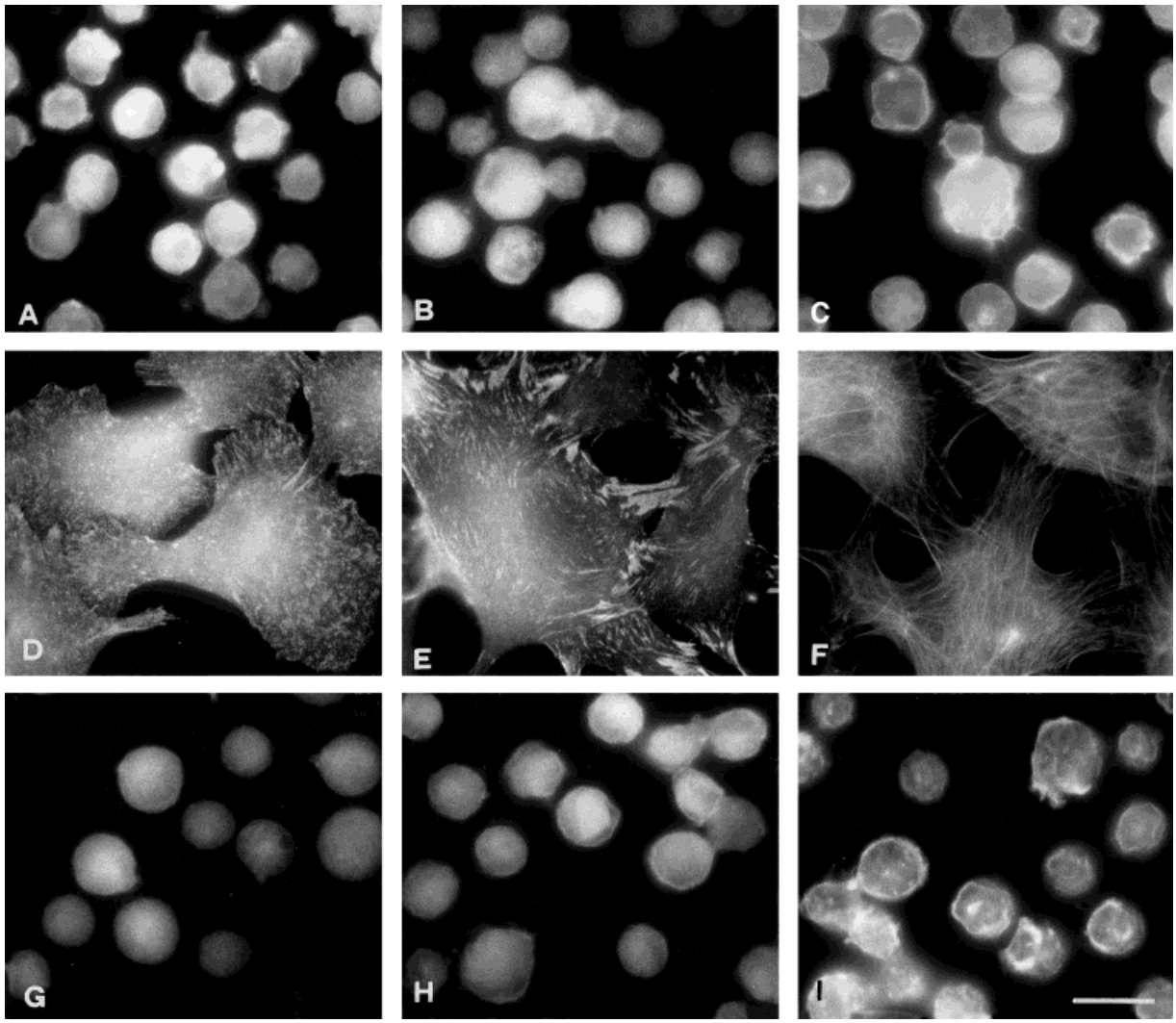


**Fig. 5.** Distribution of focal adhesion proteins in the attached rounded-up cells induced by SCE. Cells were initially attached to uncoated cover-glasses for 30 min, then incubated for 1 h with serum-free MEM (**A–C**), vitronectin (5 μg/ml; **D–F**), or both SCE (300 μg/ml) and vitronectin (5 μg/ml; **G–I**). The cells were then stained with anti-$\alpha_v\beta_3$ antibody (A,D,G), anti-vinculin antibody (B,E,H), or phalloidin-FITC (C,F,I). Scale bar = 20 μm.

tivation of FAK, and activation of FAK alone is not sufficient to promote the formation of F-actin and FAs. Taken together with the finding that FAK activation is not responsible for the increase in FA-mediated adhesion, these results suggest that a potential upstream tyrosine kinase is involved in FA formation. Nevertheless, the possibility that FAK involvement may depend on the cell type involved should be taken into consideration.

Another factor that mediates the assembly of stress fiber and FAs is the small GTP-binding protein, Rho. Microinjection of Rho into quiescent Swiss 3T3 fibroblasts induces the formation of stress fibers and FAs, which can also be

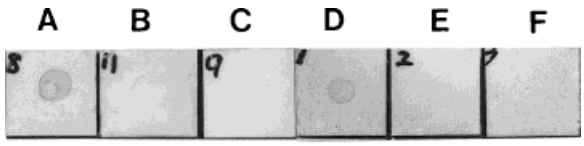


**Fig. 6.** Dot binding immunoassay for binding of SCE to integrin or vitronectin. In positive and negative controls, integrin $\alpha_v\beta_3$ (A–C) or vitronectin (D–F) was directly dotted onto the membrane strip, then incubated with anti-integrin $\alpha_v\beta_3$ antibody (**A**), anti-vitronectin antibody (**D**), or TBS-Tween (**B,E**). In the test itself, SCE was dotted onto the membrane strips, which were then treated with integrin $\alpha_v\beta_3$ (**C**) or vitronectin (**F**). Binding between SCE and integrin or vitronectin was tested by immunoblotting with anti-integrin $\alpha_v\beta_3$ (C) or anti-vitronectin (F) antibody.

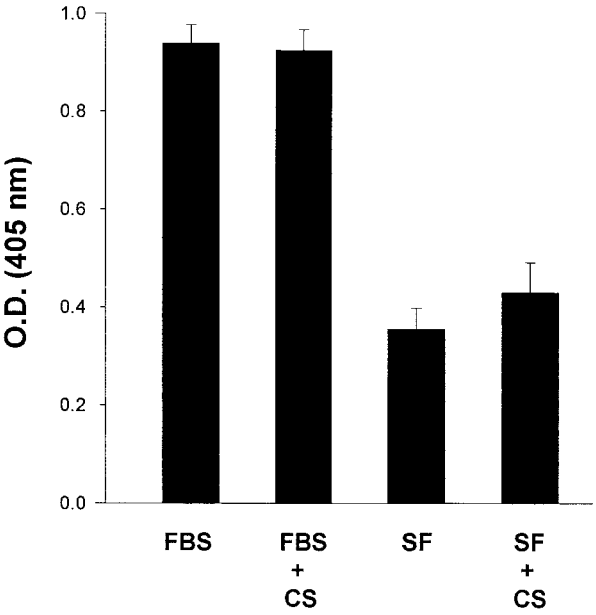


**Fig. 7.** Effect of chondroitin sulfate on cell adhesion. CPAE cells were plated in serum-free medium (SF), serum-free medium plus chondroitin sulfate (1 mg/ml; SF + CS), medium containing 10% fetal bovine serum (FBS), or medium containing 10% FBS plus chondroitin sulfate (1 mg/ml; FBS + CS).

induced by several agents, including serum [Ridley and Hall, 1992; Hall, 1998]. Recent results demonstrate that both stress fiber and FA formation can be enhanced by Rho-kinase [Amano et al., 1997], which is regulated by Rho [Nobes and Hall, 1995; Aelst and D'Souza-Schorey, 1997], and that activation of Rho-kinase is sufficient for the formation of stress fibers and FAs. In CPAE cells, the SCE-induced inhibition of cell adhesion and redistribution of FAs and stress fibers may not be mediated by inhibition of Rho activity, since actin polymerization, which is regulated by Rho [Nobes and Hall, 1995], was not prevented by SCE treatment. However, Rho may still be activated by SCE treatment of CPAE cells, since SCE-conditioned medium contains serum, and this may explain why actin filaments were polymerized under these conditions. Furthermore, stress fibers in SCE-treated, but still attached, CPAE cells were disrupted into irregular short arrays of actin filaments. We therefore propose that SCE may somehow act on a downstream target of Rho, thus interfering with stress fiber and FA formation. The possibility that SCE affects other FA proteins, such as α-actinin, which forms the link between actin filaments and FAs, cannot be excluded. Another consideration is the role of myosin light chain phosphorylation, since actin-myosin contraction is suggested to regulate the formation of stress fibers and FAs in smooth muscle cells and fibroblasts [Burridge and Chrzanowska-Wodnicka, 1996].

The SCE-induced reorganization of stress fibers and FAs cannot be attributed to the fact that SCE mimics ECM components and competes for binding sites on the cell surface, since SCE did not bind to integrin $\alpha_v\beta_3$. This conclusion is further supported by the fact that cell attachment was not affected by preincubation of CPAE cells with SCE, which also rules out the possibility that SCE may react with adhesion molecules on cell membranes other than integrins, such as cell-associated heparan sulfate proteoglycan [LeBaron et al., 1988]. Although the inhibitory effect of SCE on cell attachment was not caused by its binding to vitronectin, it may be possible that SCE sterically interferes with the interaction between cell surface receptors and extracellular substrates to prevent cell attachment. The interaction of cartilage proteoglycan with collagen [Rich et al., 1981] and the 105 kD fibronectin fragment [Bidanset et al., 1992] has been reported to inhibit cell adhesion. In contrast, preincubation of cells with proteoglycans [Knox and Wells, 1979] inhibits cell attachment, and thrombospondin has been shown to bind to heparan sulfate at the surface of bovine aortic endothelial cells [Murphy-Ullrich and Höök, 1989] and reduce the number of FAs. Thus, different agents may exert their effects on cell-substratum adhesion by binding either to cell surface receptors or to extracellular substrates. In addition, the reason why shark cartilage chondroitin sulfate did not inhibit CPAE cell adhesion may be that intact proteoglycans, but not the core proteins or glycosaminoglycans alone, are responsible for this inhibitory effect [Knox and Wells, 1979; Yamagata et al., 1989; Bidanset et al., 1992].

In summary, our results show that shark cartilage both induces cell detachment by reorganization of FAs and stress fibers and interferes with cell attachment and spreading by decreasing FA formation and preventing stress fiber formation. These effects are not due to the binding of components of shark cartilage to either integrin $\alpha_v\beta_3$ or vitronectin. The exact mechanism by which shark cartilage affects

the cell-substratum interaction is currently under investigation.

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