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Inhibition of Cell Adhesion by Type V Collagen

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ABSTRACT. **Human umbilical vein endothelial cells grew well in dishes coated with collagen types I, II, III, or IV. However, the same cells tended to detach themselves from dishes coated with type V collagen, and cell proliferation in these dishes was inhibited. Such anti-adhesive activity was partially retained by heat-denatured type V collagen or by its α1 chain, but not by its α2 chain. Several other cell types did not adhere to the type V collagen substratum even in the presence of 10% serum. The cell types strongly inhibited from adhering by type V collagen included Swiss mouse 3T3 cells and their MSV-transformants, BALB/c 3T3 cells and their methylcholanthrene-transformants, NIH 3T3 cells and their *ras*-transformants, BHK cells, CHO-9 cells, CHO-K1 cells, and mouse melanoma B16-F10 cells. Using Swiss mouse 3T3, we studied the effects of type V collagen on cell adhesion to fibronectin in serum-free medium. When the culture dishes were coated with a mixture of fibronectin with various concentrations of type V collagen, the adhesion of the cells was inhibited depending on the concentration of type V collagen. The inhibition of cell adhesion by type V collagen was competitively overcome by increased concentrations of fibronectin. The activity that interferes with the effects of fibronectin was retained mainly by the α1 chain of heat-denatured type V collagen.**

Adhesion to the extracellular matrix is a basic step in the proliferation of many types of animal cells. Cell-adhesive glycoproteins such as collagens, gelatin, and fibronectin have been generally used as the substratum for cell culture (13). For instance, capillary endothelial cells can be cultivated in dishes coated with gelatin (7, 8). Fukuda *et al.* (9) examined the abilities of different types of collagen to support the proliferation of human umbilical vein endothelial (HUVE) cells, and reported that type V collagen selectively inhibits the growth of these cells, but that collagen types I, III, or IV or fibronectin enhance cell proliferation. Madri *et al.* (17) reported that capillary endothelial cells isolated from rat epididymal fat pad proliferate in dishes coated with heterologous type I/III collagens, but that the cells do not proliferate, but form tube-like structures, on a substratum of type IV/V collagens. Staatz *et al.* (24) reported that the membrane glycoprotein complex Ia-IIa of platelets adheres to type I, II, III, and IV collagens, but that it does not adhere to type V collagen. These results suggest that type V collagen is different from other types in its cell-adhesive properties.

Type V collagen was first isolated from human placenta (1) and adult skin (3), but it has now been found as a minor collagen component in many kinds of tissues, including blood vessels (6, 22). Of the different types of collagen, only type V collagen selectively binds thrombospondin (18), DNA (10), heparan sulfate proteoglycans (15), and heparin (27). Type V collagen binds insulin while retaining its mitogenic activity (28). Both the heparin- and insulin-binding sites are in the same 30-kDa CNBr fragment of the α1 (V) chian (27, 28). Selective inhibition of the proliferation of endothelial cells by type V collagen seems to be characteristic of this collagen (9, 17). We studied the mechanisms of growth inhibition by type V collagen, and report here that the inhibition of the proliferation of HUVE cells was due to the poor adhesion or spreading of the cells on a substratum of type V collagen, and that the anti-adhesive activity of type V collagen was mainly in the α1 chain. Type V collagen inhibited the adhesion of several other cell types, too, and it interfered with the adhesion of Swiss mouse 3T3 cells to fibronectin.

MATERIALS AND METHODS

Materials. The following materials were used: bovine type I, II, III, IV, and V collagens (Koken, Tokyo), gelatin (Difco, Detroit, MI), and BSA (RIA grade; Sigma, St. Louis, MO). Fibronectin was purified from human plasma by gelatin affinity chromatography (4). The α1 and α2 chains of type V collagen were purified with a column of DEAE Toyopearl 650S (Tosoh, Tokyo). Briefly, type V collagen was dissolved at 10 mg/ml in 20 mM Tris-HCl buffer that contained 6 *M* urea, pH 8.6, and was heated at 55°C for 15 min. The sample was put on the column and eluted with a linear gradient of NaCl

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concentrations from 0 to 0.5 *M* (19). The purity of the separated chains was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Cells. HUVE cells and a growth factor supplement isolated from bovine brain were supplied by Dr. K. Kaji, Tokyo Metropolitan Institute of Gerontology. The cells were cultivated in MCDB104 GK medium (Kyokuto Pharmaceutical Co., Tokyo) that contained 10% fetal bovine serum (Nesco Bio), 100 ng/ml growth factor supplement, and 100 μg/ml heparin (Sigma) in dishes coated with 1 mg/ml collagen in 50 m*M* acetic acid at room temperature for 1 hr or at 4°C overnight. Other cell lines were from the stock of our institute and were maintained in Dulbecco's modified Eagle's minimum essential medium (D-MEM, Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), if not otherwise stated. Thymidine incorporation was assayed by the addition of 0.1 μCi/ml [methyl-^{3}H]thymidine (87 Ci/mmol, Amersham, Buckinghamshire) to the culture medium, and the radioactivity incorporated into the acid-insoluble fraction was counted with a Beckman LS 3081 liquid scintillation counter.

Cell adhesion assay under serum-free conditions. Nunclon 24-well multiplates (Nunc, Roskilde) were coated with a solution of collagen, fibronectin, or a mixture of the two at room temperature for 2 hr, and any uncoated surface of the wells was blocked by the addition of 2% BSA followed by incubation at 37°C for 2 hr. Trypsinized cells were suspended in a small volume of serum-free D-MEM that contained 0.5 mg/ml soybean trypsin inhibitor (Sigma), and then diluted appropriately with serum-free D-MEM. Approximately 10^5 cells in 1 ml of the culture medium were put in each well, and the number of attached cells was counted by means of a Coulter Counter Model D after incubation of the cells at 37°C for 1 hr.

RESULTS

Attachment and growth of HUVE cells on type V collagen. We first examined the growth of HUVE cells in dishes coated with type I, II, III, IV, or V collagens, and confirmed the report of Fukuda, *et al.* (9) that type V collagen severely inhibits the growth of HUVE cells but that other types of collagen do not (data not shown). We also confirmed that heat-denatured type V collagen retains some inhibitory activity. We then examined the effects of type V collagen or its α1 and α2 chains on the growth rate and thymidine incorporation of HUVE cells. The cells grew well on type I collagen or on the α2 (V) chain, but growth was inhibited on type V collagen or on the α1 (V) chian (Fig. 1A). The rate of incorporation of [^{3}H]thymidine into the cells on type V collagen was less than that on type I collagen or on the α2 (V) chain (Fig. 1B). On the α1 (V) chain substratum, the rate of thymidine incorporation was the same as that on type I collagen during the first 2 days of incubation, but the rate declined thereafter.

During the experiments, we noticed that HUVE cells could first attach themselves to type V collagen or to the α1 (V) chain, but spread rather poorly on them, and tended to detach themselves from the dishes after cultivation of the cells for 3 days or more. Figure 2 shows photographs of HUVE cells cultivated for 24 hr and 72 hr on different substrata. The cells remained attached to type V collagen or the α1 (V) chain at 24 hr of culture, but they became round after 72 hr, and detached themselves from the dishes after prolonged cultivation. However, on type I collagen or the α2 (V) chain, the cells were still attached after 72 hr of cultivation, and they continued to grow until confluence was reached. Human and bovine type V collagens had similar effects on the adhesion of HUVE cells. From these results, we concluded that the growth inhibition of HUVE cells by type V collagen is due to poor adhesion and eventual detachment of the cells from the substratum, and that the α1 chain of type V collagen is mainly responsible for the inhibition.

Attachment of other cell types to type V collagen. We examined the adhesion of various cell types to the type V collagen substratum in culture medium that contained 10% fetal bovine serum. The poor adhesiveness or spreading on type V collagen was not restricted to HUVE cells; the adhesion of many other of the cell types tested was more or less inhibited by type V collagen even in the presence of 10% serum. The cell types whose adhesion was severely inhibited by type V collagen included: Swiss mouse 3T3 cells and their MSV-transformants, BALB/c 3T3 cells and their methylchol-

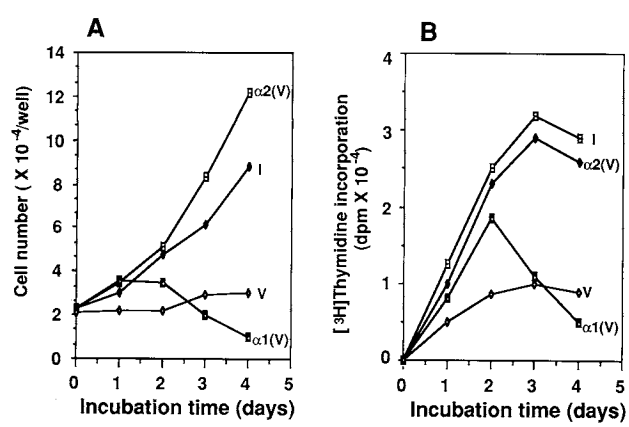

Fig. 1. Growth curve and thymidine incorporation of HUVE cells on various collagen substrata. The cells were cultivated in dishes coated with 1 mg/ml collagen type I or type V or with the α1 (V) or α2 (V) chain. Cell numbers were counted every day (A). The rate of DNA synthesis was assayed by the addition of 0.1 μCi/ml [^{3}H]thymidine to the culture medium when the cells were put in the wells. The radioactivity incorporated into the acid-insoluble fraction was measured every 24 hr (B).

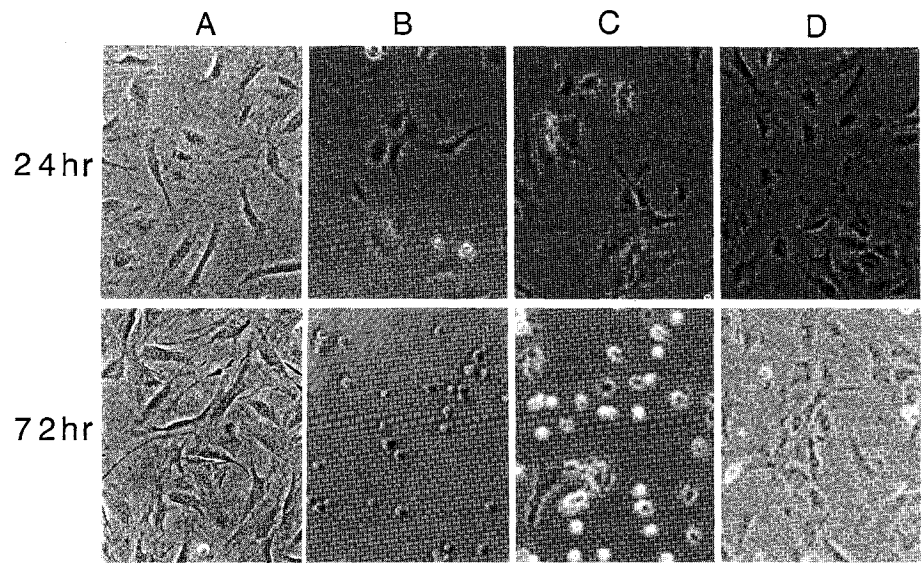

Fig. 2. Attachment of HUVE cells cultivated on various collagen substrata. The dishes were coated with 1 mg/ml type I collagen (A) or type V collagen (B) or with the α1 (V) (C) or α2 (V) (D) chain. Photographs were taken after incubation of the cells for 24 hr and 72 hr.

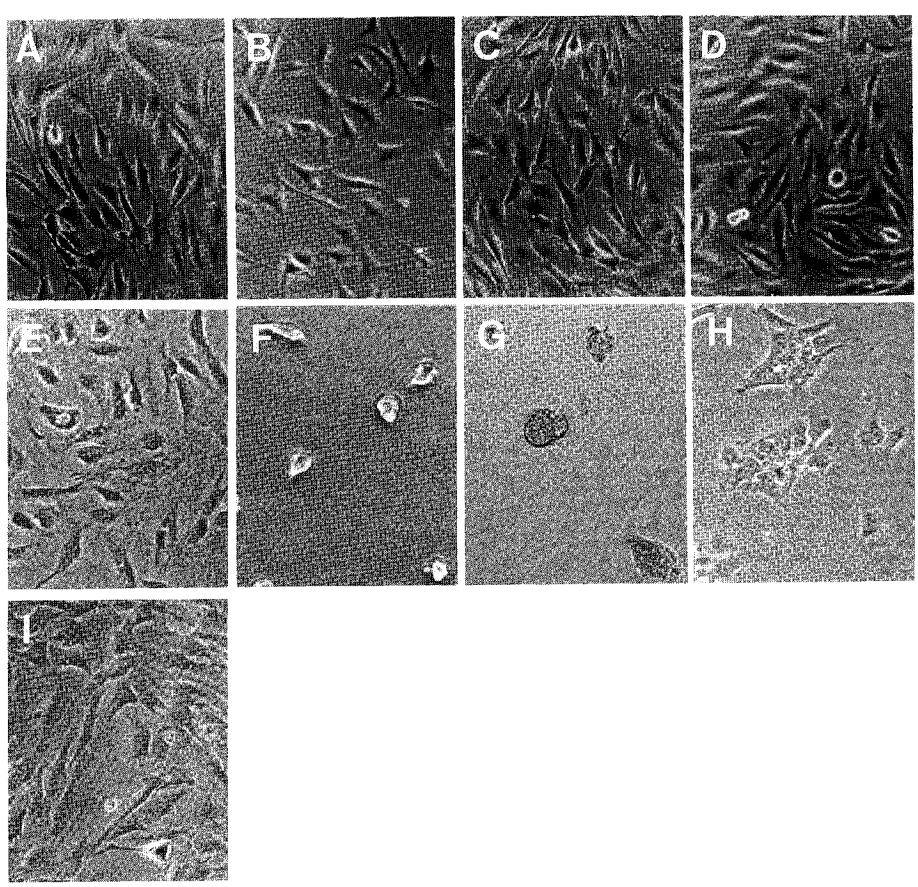

Fig. 3. Attachment of Swiss mouse 3T3 cells to various collagen substrata. The dishes were coated with 1 mg/ml gelatin (A), collagen of type I (B), II (C), III (D), IV (E), or type V (F), heat-denatured type V (G), α1 (V) (H), or α2 (V) (I). The cells were incubated at 37°C for 15 hr in D-MEM that contained 10% fetal bovine serum.

anthrene-transformants, NIH 3T3 cells and their *ras*-transformants, BHK cells, CHO-9 cells, CHO-K1 cells, and mouse melanoma B16-F10 cells. As an example, the adhesion of Swiss mouse 3T3 cells to various substrata in the presence of 10% fetal bovine serum is shown in Fig. 3. These cells attached and spread well on gelatin and on collagen types I, II, III, and IV. In dishes coated with type V collagen, however, the cell-substrate adhesion was inhibited and the cells aggregated with each other. Such an antiadhesive effect was partially retained by heat-denatured type V collagen and its α1 chain, but not by its α2 chain. Figure 4 shows the numbers of Swiss mouse 3T3 cells that attached to various collagen substrata in the presence of 10% fetal bovine serum. The attachment of the cells was repressed by about 75% at 0.5 mg/ml type V collagen, but the other types of collagen did not have this effect.

The adhesion of several other cells, such as NRK cells, HeLa cells, or human WI-38 fibroblasts, was not inhibited significantly by type V collagen. As an example, the adhesion of NRK cells to various collagens is shown in Fig. 5. These cells attached and grew in dishes coated with 1 mg/ml type V collagen as well as on the other types of collagens.

Antiadhesive activity of type V collagen. Using Swiss mouse 3T3 as a model system, we next examined the effects of type V collagen on the attachment of cells to fibronectin. Culture dishes were coated with a mixture of 10 μg/ml fibronectin and various concentrations of collagen, and the attachment of the cells in serum-free D-MEM was assayed. Type V collagen inhibited the attachment of the cells by about 60% at the concentration of 2 μg/ml, but the number of attached cells increased to about 60% of the control at 4 μg/ml, and decreased again at higher concentrations (Fig. 6A). The reason was not clear, but this biphasic effect of type V collagen was reproducible. Heat-denatured type V collagen also inhibited the attachment of the cells to fibronectin. In contrast, the cell attachment was either not inhibited or was slightly increased by similar concentrations of type I collagen.

We next examined the effects of α1 (V) and α2 (V)

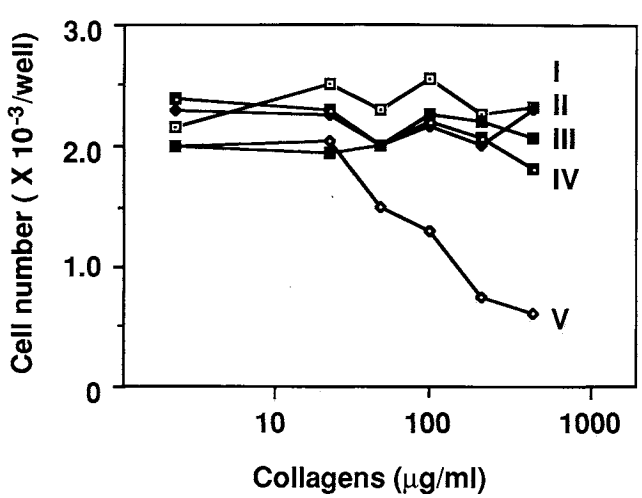

Fig. 4. Effects of collagens on the attachment of Swiss mouse 3T3 cells. Nunclon 24-well plates were coated with various concentrations of different types of collagen as indicated by the Roman numerals. The cells were inoculated at 5×10^4 cells/well in 1 ml of D-MEM that contained 10% fetal bovine serum, and the cell numbers were counted after incubation of the cells at 37°C for 2 hr.

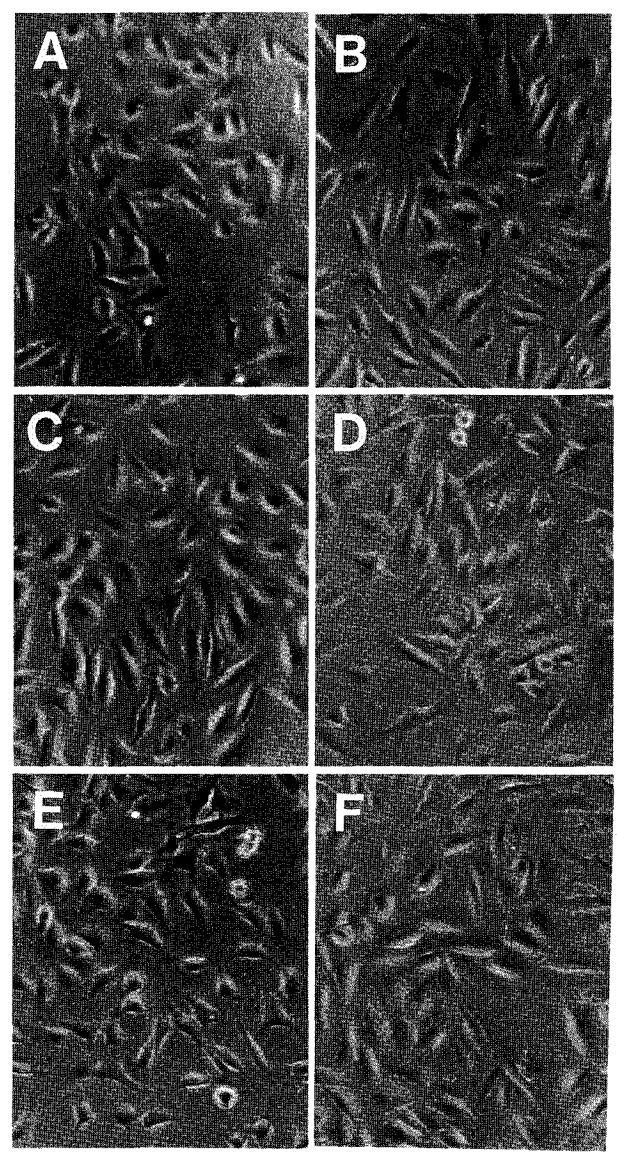

Fig. 5. Attachment of NRK cells to various collagen substrata. The experimental conditions were the same as described in the legend of Fig. 3. A, Gelatin; B, collagen type I; C, type II; D, type III; E, type IV; and F, type V.

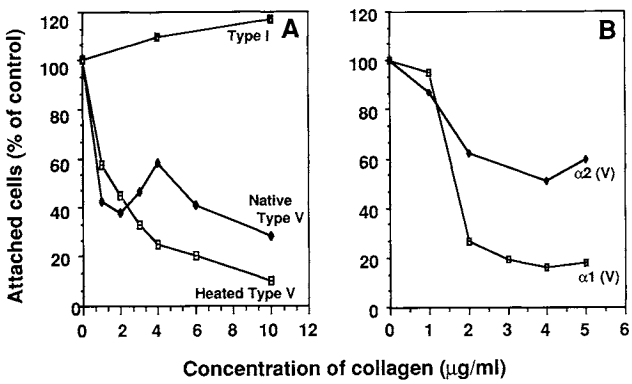

Fig. 6. Effects of type V collagen on the attachment of Swiss mouse 3T3 cells to fibronectin in serum-free medium. Nunclon 24-well plates were coated with mixtures of 10 μg/ml fibronectin and various concentrations of native or heat-denatured type V collagen, α1 (V) chain, or α2 (V) chain. The numbers of attached cells were counted after incubation of the cells in serum-free D-MEM at 37°C for 1 hr.

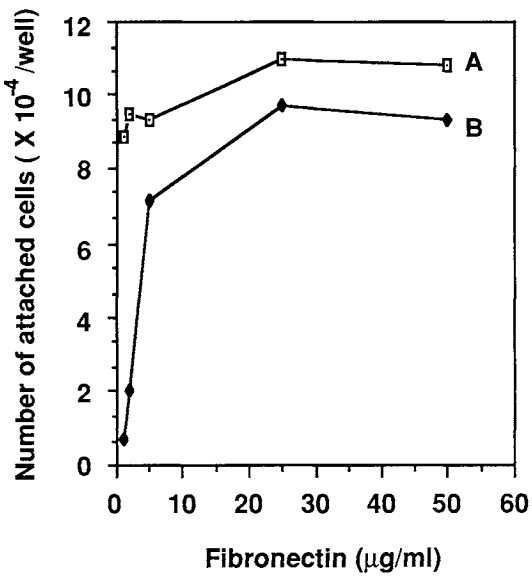

Fig. 7. Effects of increasing concentrations of fibronectin on the anti-adhesive activity of type V collagen on Swiss 3T3 cells. The wells were coated with various concentrations of fibronectin (A) or with a mixture of fibronectin and 2 μg/ml type V collagen (B). The cell attachment assay was performed as described in the legend of Fig. 6.

chains on the adhesion of Swiss mouse 3T3 cells. The attachment of the cells was inhibited by more than 80% by 3 to 5 μg/ml of the α1 (V), but was inhibited by about 50% by the similar concentrations of α2 (V) (Fig. 6). We concluded that the antiadhesive activity of type V collagen was mainly in the α1 chain.

Swiss mouse 3T3 cells were suspended in D-MEM that contained 100 μg/ml each of BSA, type I collagen, or type V collagen, and incubated at 37°C for 1 hr. The cells were then centrifuged and washed three times with D-MEM, and they were put in dishes coated with 10 μg/ml fibronectin, followed by incubation at 37°C for 1 hr. The concentration of type V collagen used in this experiment was much higher than those used in the coating of dishes to prevent cell adhesion, but pretreatment of the cells with type V collagen did not affect the adhesion of the cells to fibronectin-coated dishes (data not shown). It was therefore unlikely that the inhibition of cell adhesion was caused by the binding of type V collagen to the cell surface.

We next examined the effects of increasing concentrations of fibronectin on the antiadhesive effect of type V collagen. In this experiment, the dishes were coated with a mixture of 2 μg/ml type V collagen and different concentrations of fibronectin, and the attachment of Swiss mouse 3T3 cells was assayed. Attachment was inhibited by about 80% when the ratio of fibronectin to type V collagen was 1 : 1, but the number of attached cells increased to about 90% of the control at the ratio of 10 : 1 or more (Fig. 7). These results indicated that type V collagen competitively inhibited the cell-adhesive activity of fibronectin.

DISCUSSION

We confirmed the report of Fukuda *et al.* (9) that the proliferation of HUVE cells is inhibited by type V collagen and that this inhibition is due to the detachment of the cells from the substratum during cultivation. The antiadhesive activity was partially retained by heat-denatured type V collagen and mainly by its α1 chain. Type V collagen also inhibited the adhesion of several other cell types, such as Swiss mouse 3T3 cells, even in the presence of 10% serum. This activity was retained mainly by the α1 chain of heat-denatured type V collagen and partially by the α2 (V) chain. In contrast, the adhesion of some other cell types, such as NRK cells, was not inhibited by collagen V. Fukuda *et al.* (9) also reported that the proliferation or adhesion of human smooth muscle cells or nasal mucosa fibroblasts is not affected by type V collagen. Grotendorst *et al.* (12) reported that smooth muscle cells adhere to type V collagen, and later it was suggested that cell surface glycoproteins or glycosaminoglycans may be involved in the interaction between the cells and type V collagen (15, 16). Our results imply that the cell types strongly inhibited from adhesion by type V collagen lack specific receptors for this collagen, and also that type V collagen might interfere with the functions of cell-adhesive glycoproteins contained in the serum. This latter possibility was tested with Swiss mouse 3T3 cells and fibronectin, and we

found that the adhesion of the cells to fibronectin was inhibited by type V collagen, heat-denatured type V collagen, or the α1 (V) chain.

Collagens have been thought to mediate cell adhesion either directly (13) or through interactions with fibronectin (11, 21). However, our results indicate that type V collagen is a negative regulator of fibronectin action. Such negative control of cell adhesion by the components of the extracellular matrix has been reported by several investigators. Chiquet-Ehrismann *et al.* (2) showed that the adhesion of chick embryo or rat fibroblasts or of mouse L929 cells to fibronectin is inhibited by tenascin. This antiadhesive activity of tenascin is governed by the N-terminal domain of the molecule that contains the EGF-like repeats (23). Lahav (14) reported that thrombospondin inhibits the adhesion of bovine aortic endothelial cells to fibronectin. Yamagata *et al.* (26) showed that a large chondroitin sulfate proteoglycan synthesized by cultured chick embryo fibroblasts inhibits the adhesion of various types of cultured cells to the substratum. Nagata *et al.* (20) reported that a high concentration of soluble collagen type I inhibits the spreading of BHK cells on fibronectin. In several biological processes such as cell division, cell migration, development, and wound healing, there seems to be inhibitory regulation of cell-matrix adhesion. Modulation of cell adhesion by certain matrix molecules might occur. Type V collagen is found in a wide variety of tissues (6, 22), so its role in the regulation of the cell-matrix adhesion may be physiologically important. In this regard, the observations by Stenn et al. (25) are of note. They reported that epidermal cells in epithelial outgrowths contain type V collagen but not types I, II, III, or IV, and that continual synthesis and secretion of collagen V are necessary for migration of the epidermal cells.

The mechanisms by which type V collagen interferes with fibronectin action are not known. One possible mechanism is that the binding of type V collagen or its α1 chain to fibronectin may affect the cell-adhesive activity of the fibronectin molecule. Similar mechanisms have been postulated for the interference with cell adhesion by tenascin (2, 23) and thrombospondin (14), because these matrix proteins interact with fibronectin. However, Engvall *et al.* (5) reported that the affinity of type V collagen for fibronectin is lower than those of collagen types I, II, and III, and our preliminary observations also suggested that the specific binding of type V collagen or its α1 (V) chain to fibronectin was not necessary for the inhibition of cell adhesion. Detailed studies on the mechanisms of the antiadhesive effect of type V collagen are now in progress.

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