Contact with Basement Membrane Heparan Sulfate Enhances the Growth of Transformed Vascular Endothelial Cells, but Suppresses Normal Cells

Toru Imamura¹, Yoshihito Tokita and Youji Mitsui

Cell Science and Technology Division, Fermentation Research Institute, Higashi 1-1-3, Tsukuba Science City 305, Japan

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ABSTRACT. Modulation of vascular endothelial cell growth by basement membrane heparan sulfate was investigated using four lines of normal and transformed cells. The growth of transformed endothelial cells, but not normal cells, on reconstituted basement membrane was severely suppressed when heparan sulfate, one of the components of the membrane, was specifically degraded by an enzyme, heparitinase. Similarly, when cells were grown on surfaces coated with heparan sulfate, as little as 60 pg/cm² of heparan sulfate caused growth enhancement of transformed cells, but suppression of normal cells. These results together with our previous observations (IMAMURA, T and MITSUI, Y. (1987) Exp. Cell Res., 172: 92-100) argue that transformed cells have reversed a mechanism by which basement membrane heparan sulfate functions as a physiological suppressor for the growth of normal endothelial cells.

We have previously shown that heparan sulfate added in culture media suppresses normal endothelial cell growth but potentiates transformed cell growth (5). We have also reported a line of evidence suggesting that heparan sulfate is deeply involved in the contact inhibition of normal cell growth (8, 9). Although it has been suggested by others and ourselves that heparan sulfate or heparin directly affects various cell behavior, e.g., growth, differentiation and morphology (2), and metabolism (15), it still needs to be directly proven.

In the milieu surrounding endothelial cells, several forms of heparan sulfates exist either as free sugar chains or proteoglycans in the basement membranes, extracellular matrix, and cell membranes. Cell membrane-associated heparan sulfate proteoglycans are suggested to function as molecules that cross-link the cells to the extracellular matrix proteins, such as fibronectin (7) and laminin. The involvement of these heparan sulfates, however, in the growth regulation of endothelium has not been intensively investigated.

Since normal human vascular endothelial cells usually require heparin-binding growth factors (HBGFs) in the culture media, heparan sulfate or heparin as soluble forms can modify cell growth through interaction with the growth factors (5, 6), which makes it difficult to evaluate the direct action of these glycosaminoglycans (GAGs) on cell growth. In the present study, by utilizing refined experimental conditions, we report that heparan sulfate in reconstituted basement membranes is capable of suppressing the normal endothelial cell growth via substrate-cell interactions, and enhancing the growth of transformed cells.

MATERIALS AND METHODS

Cells. Normal vascular endothelial cells: Human umbilical vein endothelial (HUE) cells were isolated from human umbilical vein and cultured in MCDB151 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 15% heat-inactivated fetal bovine serum (FBS; Bocknek Laboratories Inc., Canada), 5 ug/ml heparin (Sigma) and crude endothelial cell growth factor (1), as previously described (5). HUE cells had a life span of 78 population doubling level (PDL) under these conditions and were used between 15 and 40 PDL in the present study. Bovine aortic endothelial (BAE) cells were isolated from bovine aorta and cultured in Dulbecco’s modified minimum essential medium (DMEM; Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% FBS. BAE cells had a lifespan of about 90 PDL and were used between 40 and 55 PDL in this study. Normality of both HUE and BAE cells were verified by the number and integrity of their chromosomes.

Transformed endothelial cells: tHUE2 cells were cloned from spontaneously transformed HUE cells, ECV304 (14). They were established as an immortalized cell line with an ability to grow in a serum-free medium (Kobayashi et al., manuscript in preparation). tHUE2 cells were cultured in ASF301 medium (Ajinomoto Co, Tokyo, Japan) and used be-
between 210 and 250 PDL. PAE-20 cells were derived from a primary culture of porcine aorta endothelial cells and had spontaneously transformed to an immortalized cell line, as previously described (11). PAE-20 cells were cultured in DMEM medium supplemented with 10% FBS and were used between 220 and 250 PDL.

**Cell growth on enzyme-treated, basement membrane-type matrix.** Three hundred microliters of basement membrane extract (Matrigel; Collaborative Research Inc., Lexington, MA) diluted 1:10 in DMEM medium was poured at 4°C into each well of a 24-well plate (Becton Dickinson Labware, Oxford, CA) and incubated at 37°C for 1 h to complete the gelation of Matrigel. Each well was washed once with 1 ml of PBS (10 mM phosphate-buffered saline, pH 7.3), then treated at 37°C for 150 min with 300 μl of one of the following enzyme solutions: heparitinase (Seikagaku Kogyo, Tokyo, Japan) 17 CU/ml, collagenase (Sigma) 17 CU/ml in 10 mM Tris-HCl, 0.5 mM CaCl2; trypsin (culture grade; Handai Biken, Osaka, Japan) 0.25% in PBS. Control wells were treated with 300 μl of PBS. After the digestion, each well was washed four times with 1 ml of PBS. Cells in their respective medium were seeded to the well at a density of 2 × 10^4/cm², and incubated at 37°C in 5% CO₂ for 6 h (18 h for tHUE2 cells) until all the viable cells were attached. To estimate the attached cell number, one group of wells was rinsed with 0.02% EDTA in PBS. The cells were then detached with 0.2 ml of trypsin (0.25% in PBS), and counted in a hemocytometer. The other group of the cells was washed once, refed with fresh medium, and then incubated for 48 h (56 h for BAE cells). After the incubation, the cell numbers were counted as described above.

**Coating dishes with heparan sulfate.** Thirty-five mm diameter dishes (Corning) containing 1 ml of heparan sulfate (from bovine kidney; Seikagaku Kogyo solution in saline were incubated for 24 h at room temperature to allow the inner surfaces of the dishes to adsorb heparan sulfate. The dishes then received three washes with 1 ml of saline and one additional wash after incubation at 37°C for at least 6 h.

**Cell growth on heparan sulfate-coated dishes.** Cells were plated to the dishes coated with various concentrations of heparan sulfate directly and to confirm its inverse effect on the growth-regulatory action of basement membrane heparan sulfate proteoglycan, collagen type IV, and other proteins including laminin and entactin, it was digested before cell inoculation with respective degrading enzymes to interpret the involvement of each component in the growth regulation; heparitinase, collagenase or trypsin was used. To evaluate cell proliferation on the treated Matrigel excluding the possible differences in the attached cell numbers, cell growth was expressed by the proliferation index as calculated by the formula:

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\text{Proliferation Index} = \frac{(\text{cell number at the end of culture/attached cell number}) - 1}{\text{cell number}}
\]

The plating efficiency varied from 45% (tHUE2 cells) to 95% (PAE-20 cells) depending on the cell type, but the differences among the enzyme treatments were less than 10% for each cell type. The proliferation indexes were presented as percentages of control. It may be noteworthy that all the cells, except tHUE2 cells, grew slowly on the control Matrigel; PAE-20, tHUE2, BAE, and HUE cells respectively underwent 1.0, 3.2, 2.3, and 0.6 population doublings during the culture.

When the growth of transformed vascular endothelial cells was examined, significant suppression of their growth was observed on the heparitinase-treated matrix. Significance of the growth suppression for PAE-20 cells (Fig. 1A) and tHUE2 cells (Fig. 1B) was verified by Fisher’s Protected Statistical Determinations (p-value = 0.0053 and 0.0015, respectively). Microscopic inspection of the matrix structure did not reveal any obvious change. Collagenase and trypsin treatment also caused growth suppression of tHUE2 cells (Fig. 1B), but not PAE-20 cells (Fig. 1A).

On the other hand, when normal endothelial cells with limited lifespans were examined, heparitinase treatment of the Matrigel exerted different effects; BAE cells (Fig. 1C) and HUE cells (Fig. 1D) were not growth-suppressed by heparitinase treatments of the matrix at all, in contrast with the transformed cells.

These results imply that heparan sulfate in the basement membrane acts as an inverse growth modulator to normal and transformed cells. In order to test the growth-regulatory action of basement membrane heparan sulfate directly and to confirm its inverse effect on normal and transformed cells, we analyzed the growth of endothelial cells on plastic surfaces coated with various concentrations of heparan sulfate. This effort excluded the effects of heparitinase treatment to disanchor heparansulfate-binding molecules in the matrix, such as fibronectin and collagen.

**RESULTS**

**Vascular endothelial cell growth on Matrigel and effects of enzyme treatments.** In order to gain insight into the role of basement membrane components in regulating the growth of vascular endothelial cells, cell growth on reconstituted basement membrane matrix, Matrigel, was examined. Since the matrix is composed of heparan sulfate proteoglycan, collagen type IV, and other proteins including laminin and entactin, it was digested before cell inoculation with respective degrading enzymes to interpret the involvement of each component in the growth regulation; heparitinase, collagenase or trypsin was used. To evaluate cell proliferation on the treated Matrigel excluding the possible differences in the attached cell numbers, cell growth was expressed by the proliferation index as calculated by the formula:

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Fig. 1. Normal and transformed vascular endothelial cell growth on enzyme-treated matrix. A and B, transformed (immortal) endothelial cells; A, PAE-20 cells; B, tHUE2 cells. C and D, normal diploid endothelial cells; C, BAE cells; D, HUE cells.

Cells were plated to the wells of 24-well plates coated with matrigel and treated with respective enzyme as indicated. After 6 h (18 h for tHUE2 cells), the attached cells were trypsinized, and their numbers were counted with a hemocytometer. For separate plates, after the unattached cells had been removed, the cells were fed with 1 ml of fresh medium and further cultured. After 48 h (56 h for BAE cells), the cell numbers were counted, and the proliferation index was calculated and expressed as percentages of control ± standard deviations (as described in results). Similar results were obtained in three independent experiments.

Growth of endothelial cells on heparan sulfate-coated plates. In order to immobilize heparan sulfate on the surfaces of culture dishes, the dishes were incubated with heparan sulfate solution and then washed extensively to get rid of free heparan sulfate that can escape from the surface into the medium. In addition to heparan sulfate, due to the structural and functional similarity as a growth regulator (5), heparin was also tested by the same system using radio-labeled heparan sulfate. It was found that a single wash of the inner surface of a dish incubated with heparan sulfate solution, a procedure previously used by other investigators (12), was far from sufficient to deplete the dish surface of soluble heparan sulfate (data not shown). The procedure we used involved five extensive washes of each coated dish; three washes after coating, an additional wash at 37°C for 6 h, and finally, a wash at 37°C for removal of unattached cells just before starting the culture. It was found that no heparan sulfate was freed from the dish surface into the medium after this series

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Fig. 2. Normal and Transformed Vascular Endothelial Cell Growth on Heparan Sulfate-coated Dishes

A and B, transformed (immortal) endothelial cells; A, PAE-20 cells; B, tHUE2 cells. C and D, normal diploid endothelial cells; C, BAE cells; D, HUE cells.

Cells were plated and incubated for 6 h in growth medium to 35-mm dishes previously coated with the indicated concentrations of heparan sulfate. The attached cells were trypsinized, and cell numbers were counted with a Coulter Counter. For separate dishes, attached cells were washed once and refed with 2 ml of fresh medium, and further cultured for 48 h (65 h for BAE cells). The cell numbers were then counted and cell proliferation±S.D. was expressed as in the legend to Figure 1. Similar results were obtained in three independent experiments.

The quantity of heparan sulfate immobilized on the dish surface was less than the detection limits of the Pyroll-HCl method (200 ng/ml), which is considered to be one of the methods with highest sensitivity, even when the coating solution contained as high as 200 μg/dish of heparan sulfate (data not shown). It was not able to detect it even by using 3H-labeled heparan sulfate in the coating solution. This prompted us to measure the amount of immobilized heparan sulfate by the specific binding of $^{125}$I-labeled fibronectin to the immobilized heparan sulfate on the plastic surface. It was found that the amount of fibronectin bound to each dish correlated almost linearly with the amount of heparin or heparan sulfate used for coating, up to 5 mg/ml (Imamura and Tokita, unpublished observation). Assuming that fibronectin binds to heparan sulfate (the mean molecular weight was assumed to be 20 KDa by...
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gel filtration assay; data not shown) by a molar ratio of 1:1, the absolute quantity of heparan sulfate was calculated to be 16 pg/cm² when the dish was treated with 200 μg/ml heparan sulfate, and the thus calculated values were presented in the abscissa of Figure 2.

As shown in Fig. 2A, the growth of transformed PAE-20 cells on coated heparan sulfate was significantly enhanced from 6 pg/cm² (p=0.016) up to 60 pg/cm² (p=0.004). Similarly, tHUE cells were growth enhanced (p=0.003 at 30 pg/cm²; Fig. 2B), although to a lesser extent than PAE-20 cells. The control PAE-20 and tHUE cells respectively underwent 1.7 and 2.6 population doublings during the culture period.

By contrast, heparan sulfate coating had an inverse effect on normal cells; the growth of BAE cells was significantly suppressed on heparan sulfate coated dishes (p=0.001 at 120 pg/cm²; Fig. 2C). Likewise, HUE cells were also growth suppressed (p=0.009 at 60 pg/cm²; Fig. 2D). Population doublings for BAE and HUE cells were 3.0 and 1.8, respectively.

These responses of normal and transformed cells to immobilized heparan sulfate were in good correlation with the results obtained from the reconstituted membrane experiments described above, where digestion of heparan sulfate caused growth promotion of transformed cells, but not normal cells.

DISCUSSION

Growth of vascular endothelial cells plays a key role in many pathophysiological phenomena including development, angiogenesis and wound healing; it is considered to be regulated by a variety of soluble and insoluble factors. In our previous reports, by examining the effects of soluble glycosaminoglycans, we proposed that heparan sulfate may be one of the important physiological suppressors of normal endothelial cell growth, but not of transformed cells (5, 10). The actual role of heparan sulfate as a component of basement membrane as growth modulator, however, is still unclear because it is possible that soluble heparan sulfate interacts with heparin-binding endothelial cell growth factors added in the medium, resulting in the modulation of their binding to their high-affinity receptors (6). To overcome this potential problem, experimental systems to examine the effects of immobilized heparan sulfate were developed. As a result, a line of evidence obtained in the present study implies that basement membrane heparan sulfate is in fact capable of modulating the growth of endothelial cells whereon it lies; it promotes transformed cell growth, but suppresses normal cell growth.

We developed two systems to measure the effects of basement membrane heparan sulfate on the growth of endothelial cells. One was to use the reconstituted basement membrane, Matrigel, a basement membrane-like extracellular matrix prepared from EHS tumor which contains heparan sulfate proteoglycans, collagen type IV, laminin, and entactin as its major components. When this matrix was treated with an enzyme, heparitinase, to digest heparan sulfate (degraded heparan sulfate equivalent to 42 micromoles of uronic acid), the growth of normal and transformed cells on the matrix were differently affected; growth of transformed cells were drastically suppressed, whereas that of normal cells was even slightly enhanced. Since the enzyme was very pure and specific to heparan sulfate (impurities such as proteinase was <0.001%), these effects were attributed solely to the loss of heparan sulfate from the matrix. Both collagenase or trypsin digestion exerted growth modulation on most of the cells to some extent, suggesting that each component is potentially important in growth regulation of endothelial cells; however, there were no consistent effects correlated with cellular transformation.

Another test we performed to determine the effect of basement membrane heparan sulfate was to coat the plastic culture surfaces with heparan sulfate. This approach assures, needless to say, that the effects observed are those of the molecule used for coating. In fact, in good agreement with the matrix experiments, it was shown that immobilized heparan sulfate alone enhanced the growth of transformed cells, but suppressed that of normal cells. Heparin exerted similar effects as heparan sulfate (data not shown). It is noteworthy that such small amounts of heparan sulfate or heparin could cause significant modulation of cell growth. Interestingly, by determining the concentrations of heparan sulfate on the plastic surface, it was shown that as little as 60 pg/cm² of heparan sulfate exerts significant effects on the growth of all the cells tested. Even by adding heparan sulfate to the media to these concentrations did not produce significant effects (data not shown). It is therefore suggested that the immobilized heparan sulfate is somehow being very effectively recognized by the endothelial cells, which leads to the growth modulation of the cells.

Thus, in both systems, virtually the same results were obtained; basement membrane heparan sulfate enhanced growth of transformed endothelial cells, but suppressed the growth of normal cells.

The mechanism by which basement membrane heparan sulfate regulates cell growth is at present unknown. It is possible to explain the action with heparan sulfate and heparin by binding of heparin-binding growth factor (HBGF)s. If this is the case, absorption by heparan sulfate of HBGFs contained in culture media (thus decreasing the effective concentration of HBGFs in the media) may explain the suppression of normal endothelial cell growth. However, it does not explain the growth enhancement of transformed cells. Another explanation
would be that immobilized heparan sulfate might bind HBGFs and facilitate their presentation to the transformed cells, just as in the case for hemopoiesis (4, 13). However, it does not explain the growth suppression of normal endothelial cells. It is unclear at present if there are some receptor-like molecules on the cell surface that recognize heparan sulfate and transduce some of the signals which regulate cell growth. In fact, the binding molecules for heparan sulfate and other glycosaminoglycans seem to exist on cell surfaces (Imamura et al., unpublished observation), and one of these could be the functional heparan sulfate receptor. If this is the case, then there may be a difference in the signal transduction systems between normal and transformed cells. These questions remain to be elucidated.

Considering the fact that heparan sulfate on the cell surface is likely to suppress the growth of adjacent cells, and that this action may be a part of the mechanism of contact inhibition in cell growth, it is interesting to note that the growth of the immortalized cells was enhanced by heparan sulfate, even though they maintain the capability of contact inhibition. This apparent discrepancy also awaits further investigation. Our recent finding on differential biosynthesis capacity of hyaluronic acid and heparan sulfate in normal and immortal endothelial cells (16), however, would suggest the significance of hyaluronic acid as another factor in the cell growth regulatory system.

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