Enhancement in the Adhesion of Tumor Cells to Endothelial Cells by Decreased Cholesterol Synthesis

Chittoor K. Ramachandran, Karen Sanders, and George Melnykovych

Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66103, and Research Service (151), Veterans Administration Medical Center, Kansas City, Missouri 64128

ABSTRACT

Adhesive interactions between tumor cells and the endothelial cells are presumed to be an obligatory step in the metastatic process. Using an *in vitro* model, we have examined the role of endothelial lipids in the regulation of this interaction. The cholesterol levels of bovine aorta endothelial cell monolayers were inhibited by the addition of compactin, 25-hydroxycholesterol, or 7-ketocholesterol. Metastatic B4a melanoma cells prelabeled with *14*C-amino acid mixture were then deposited on this monolayer as a suspension and, at various time intervals, the number of cells adhering to the monolayer was determined. The results indicated that inhibition of cellular cholesterol caused enhancement in cell adhesion. On the other hand, perturbations of the glycosylation in the endothelial cells were without any effect on cell adhesion. The presence of cholesterol dispersion in the growth medium partially reversed the enhancement. On the other hand, perturbations of the glycosylation in the endothelial cells prelabeled with MC-amino acid mixture were then deposited on this monolayer and, at various time intervals, the number of cells adhering to the monolayer was determined. The results indicated that inhibition of cellular cholesterol caused enhancement in cell adhesion. On the other hand, perturbations of the glycosylation in the endothelial cells were without any effect on cell adhesion. The presence of cholesterol dispersion in the growth medium partially reversed the enhancement in cell adhesion caused by the cholesterol inhibitors. Growth in the presence of retinol or dexamethasone (1 mM) also caused enhancement in the adhesiveness of the tumor cells to endothelial cells, possibly because of their effects on cholesterol synthesis. Propane, a local anesthetic which is known to increase membrane fluidity, also increased the tumor cell-endothelial interaction, suggesting that the membrane fluidity plays an important role in the regulation of cell adhesion.

INTRODUCTION

In the series of complex sequential steps that lead to the metastases of tumor cells to distant sites, interaction of tumor cells with the endothelium is an obligatory step (1). Following this interaction, specific enzymes located at the surface of the metastatic cells hydrolyze the respective matrix components of the endothelium (1). This enables the cells to traverse the endothelial barrier. There are several lines of evidence indicating that the carbohydrate residues located at the tumor cell surface are involved in their binding with the matrix components and may be responsible for the metastatic properties (2, 3). Terranova *et al.* (4), Roberts *et al.* (5), and Liotta (6) have provided evidence for the cell adhesion at the molecular level and have observed the involvement of laminin in such interactions. Recently, Dennis *et al.* (7) have identified certain oligosaccharides located at the tumor cell surface participating in the cell-cell interaction. Most of these studies have focused primarily on the properties of the tumor cell surface as the initiating factors for the cell-cell interactions. In contrast, the participation of endothelial cells in this process has not been studied in detail.

Several groups have observed that glycolipids may be involved in the interactions of different cell types (5). Particularly interesting is the observation of Huang (8), which demonstrated that the presence of glycolipids promoted the binding of erythrocytes to HeLa cells. This work also showed that the efficacy of the adhesion correlated with the glycolipids containing at least four sugar molecules with a terminal galactose. Since glycolipids are specific components of plasma membrane (9, 10), the role of plasma membrane in cell adhesion is implicated by these results. Our past work has demonstrated that alterations of plasma membrane cholesterol synthesis by several agents and conditions such as the presence of glucocorticoids, serum removal, or addition of delipidized serum to the growth medium are accompanied by changes in several membrane related functions, including endocytosis (11), alkaline phosphatase activity (12), and phospholipid methylation (13). Based on these observations, we reasoned that alterations in cholesterol synthesis may also affect adhesion properties of the cells. Little information is available on the role of cholesterol in endothelial cells in regulating the tumor-endothelial interaction. We have, in this study, addressed this question, and the results indicate that adhesion of tumor cells to the endothelial cells can be regulated by the endothelial cholesterol synthesis and is mediated through membrane fluidity.

MATERIALS AND METHODS

Cells and Cell Culture. The bovine aorta endothelial cells used in this study were first characterized by Duthu and Smith (14) and were given to us by Dr. James Smith from W. Alton Jones Cell Science Center, Lake Placid, NY. The stocks of cells in early passage were stored in liquid nitrogen, and the seed cultures were recovered when needed. The cells were grown in DMEM containing penicillin and streptomycin (100 μg/ml each) supplemented with 15% FBS. For the experiments, the cells were first inoculated in T-25 (Corning) flasks at an initial density of 5 x 10⁴ cells/flask. The cells were incubated for 24 h at 37°C before the various treatments.

The mouse B4a melanoma cell variant F10 (highly metastatic) line was obtained through the generosity of Dr. Joseph Mayo, Frederick Cancer Research Institute, Frederick, MD. The cells were cultured in monolayer with DMEM supplemented with 10% FBS. Prior to use, the cells were detached from the substratum by trypsin/EDTA and washed with DMEM. Similarly, lung carcinoma A549 cells were also grown in DMEM containing 10% FBS.

Cell Adhesion Experiments. The method for the quantitative assay for the adhesion of cells to the endothelial monolayer was based on that of Walther *et al.* (15). In a typical experiment, endothelial cells were seeded in T-25 flasks at a density of 5 x 10⁵ cells/ml in a volume of 10 ml of DMEM containing 15% FBS. As the cells reached confluency (48 h later) the medium was aspirated and replaced with 10 ml of DMEM containing 15% delipidized serum (16) with or without additional additions. The incubation at 37°C was resumed and, 24 h later, the cell adhesion assay was performed.

The neoplastic cells were removed from the monolayer by trypsinization and reseeded in DMEM to obtain a concentration of 1.5 x 10⁵ cells/ml. Ten ml of this suspension were added to the endothelial monolayer. The cultures were then incubated at 37°C with or without shaking (200 oscillations per min). At various time intervals (0–60 min), the unattached cells were counted in a Coulter Counter. In some experiments, the tumor cells were prelabeled with 20 μCi of *14*C-amino acid. The abbreviations used are: DMEM, Dulbecco's modified Eagle's minimal essential medium; FBS, fetal bovine serum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.
acid mixture (ICN, Catalog No. 1014;6; specific activity, 50 μCi/μmol) 24 h prior to removal from T-150 flasks by trypsinization. The cells were then washed three times with DMEM to remove any adhering radioactivity. The suspended labeled cells were then added to the endothelial monolayer. At the end of the incubations, the monolayers were washed three times with DMEM and dissolved in 1 ml of 0.5% sodium deoxycholate solution. Portions of the lysates (500 μl) were transferred to liquid scintillation counting vials for radioactivity measurement.

Preparation of Cholesterol Dispersions. Solutions of cholesterol (19.3 mg) and egg yolk phosphatidylcholine (36.8 mg) in chloroform were added to sonication vials and dried under nitrogen. To this, 5 ml of delipidized serum were added, and the mixture was sonicated. The sonicated mixture was centrifuged at 10,000 × g for 10 min, and the supernatant was filtered through a filter unit (0.22 μm) into a sterile tube. Dispersions containing no cholesterol were also prepared for use as controls.

RESULTS

The data presented in Fig. 1 represent our study on the binding of a lung carcinoma cell line A549 to the endothelial monolayer. The binding of tumor cells to the monolayer increased with time up to 60 min of incubation. Prior treatment of endothelial cells with compactin (2 μg/ml) for 24 h caused an increase in the tumor cell binding. Compactin treatment also resulted in a decrease in cholesterol synthesis in these cells, as indicated by the decrease in the incorporation of [14C]acetate into digoxigenin precipitable sterols (data not given). We further investigated whether or not the increased binding is associated with reductions in cholesterol synthesis by the use of agents that are known to inhibit cellular cholesterol. Accordingly, the endothelial cells were exposed to 0.1 μg/ml each of 25-hydroxycholesterol and 7-ketocholesterol for 24 h prior to the binding experiments. Both of these agents caused a stimulation in cell adhesion (Fig. 1). It was seen that at equal concentrations, 25-hydroxycholesterol was more effective than 7-ketocholesterol in stimulating cell adhesion, which is proportional to its higher ability to inhibit cholesterol synthesis in other cell types (17). The results presented in Fig. 1 also show that neither tunicamycin nor swainsonine at a 0.1 μg/ml concentration was effective in causing any change in cell adhesion.

It was important to ensure that possible adhesion of tumor cells to the exposed plastic surfaces did not interfere with the adhesion assay. We therefore examined the pattern of the cell monolayer by scanning electron microscopic techniques. Fig. 2A shows a representative sample of endothelial monolayer after the cells were grown for 24 h in delipidized serum-containing medium. Fig. 2B shows the tumor cells (B16 melanoma) adhering to the endothelial monolayer. It is clear from the figure that tumor cells adhered primarily to the endothelial cell surface and not to the plastic.

We have shown previously that in a variety of cells, dexamethasone causes inhibition of cholesterol synthesis (18). In this study, we examined the effect of this steroid on endothelial cells and its possible correlation with cell adhesion. As shown in Fig. 3, endothelial cells treated with dexamethasone for 24 h bound more A549 cells than the controls. Fig. 3 also shows that similar treatment with 1 μM retinol also caused a stimulation in cell adhesion. The possible relation between this effect of retinol and cholesterol synthesis was studied subsequently, and the result is given in Fig. 4. In the presence of delipidized serum, both retinol and retinoic acid (1 μM concentration) inhibited the incorporation of [14C]acetate into cholesterol fraction.

It is possible that conditions that cause lowered membrane cholesterol result in increased membrane fluidity, which in turn enhances cell adhesion. We examined this possibility by studying the effect of procaine (a local anesthetic known to increase the membrane fluidity in many systems) on cell adhesion. In these experiments the tumor cells used were B16 melanoma (F10). Exposure of endothelial cells to procaine caused a stimulation in cell adhesion (Table 1). Adhesion of B16 melanoma cells to the endothelial cells was similar to the A549 cells, as shown in initial experiments. Adhesion of these cells was stimulated by previous exposure of endothelial cells to compactin, dexamethasone, and retinol (Table 1). It was also noticed that the presence of cholesterol dispersion caused an inhibition in cell adhesion compared to the respective controls, which contained phosphatidylcholine dispersions. Cholesterol dispersion also reversed the compactin-induced increase in cell adhesion (Table 1). By using [14C]cholesterol in the preparation of liposomes, it was confirmed that the cholesterol molecules were incorporated into the cells during the 24 h treatment (data not given).

We confirmed the inverse relationship between cellular cholesterol and cell adhesion using compactin as a tool. In these experiments, B16 melanoma cells labeled previously with 14C-amino acid mixture were used for compactin treatment. Various agents at indicated concentrations for 24 h in the presence of 15% delipidized serum-containing medium. The carcinoma cells were suspended in DMEM and added to the cultures and, at various time points, the unattached cells were counted in a Coulter Counter. The values given are mean of six separate determinations. Bars, SD. Each point in cultures treated with compactin, 25-hydroxycholesterol, and 7-ketocholesterol was significant (P < 0.001). At all time intervals tunicamycin and swainsonine treated cultures were not different from the controls (P > 0.05). * 0.1 μg/ml; ** 2 μg/ml.
Fig. 2. Scanning electron micrographs of (A) bovine endothelial cell culture and (B) B5 melanoma cells (F10) adhering to the endothelial cell culture. Bovine endothelial cells were grown to confluency, and 24 h prior to adhesion assay they were maintained in DMEM containing 15% delipidized serum. Melanoma cells were deposited on the culture and incubated at 37°C for 15 min. Unattached cells were then removed by washing, and the cells were fixed in glutaraldehyde. The cultures were photographed in a JEOL JSM 35 scanning electron microscope. Magnification, × 900.
CHOLESTEROL AND CELL ADHESION

Fig. 3. Stimulation of cell adhesion by dexamethasone and retinol. Endothelial cells were grown as described in "Materials and Methods." Retinol (1 μM) or dexamethasone (1 μM) was added to the cultures as an ethanolic solution at the time when the medium was replaced with delipidized serum-containing DMEM. Prior to this addition, the monolayers were washed twice to remove any traces of serum lipids. Twenty-four h later the cells were used for the cell adhesion study using A549 cells. Points, mean of triplicate determinations; bars, SE.

Table 1 Effect of modification of membrane fluidity on endothelial-tumor cell binding

Endothelial cells were treated with the indicated agents for 24 h in the presence of delipidized serum-containing medium. Cholesterol dispersions or phosphatidylcholine (PC) dispersions were also added to the cultures for 24 h prior to the binding assay. The neoplastic cells used for the cell adhesion assay were B16 (F10) melanoma cells. The assay was carried out for 30 min.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cell attached (cpm/cm²)</th>
<th>% of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2355 ± 96°</td>
<td></td>
</tr>
<tr>
<td>Control + PC dispersion</td>
<td>2468 ± 86</td>
<td></td>
</tr>
<tr>
<td>Procaine (10 μg/ml)</td>
<td>2879 ± 162</td>
<td>22</td>
</tr>
<tr>
<td>Procaine (100 μg/ml)</td>
<td>3644 ± 105°</td>
<td>55</td>
</tr>
<tr>
<td>Dexamethasone (1 μM)</td>
<td>3094 ± 122°</td>
<td>31</td>
</tr>
<tr>
<td>Retinol (1 μM)</td>
<td>2974 ± 208°</td>
<td>25</td>
</tr>
<tr>
<td>Cholesterol dispersion</td>
<td>1922 ± 29°</td>
<td>-19</td>
</tr>
<tr>
<td>Compactin (2 μg/ml)</td>
<td>2969 ± 89°</td>
<td>26</td>
</tr>
<tr>
<td>Compactin + cholesterol dispersion</td>
<td>2197 ± 28°</td>
<td>-26</td>
</tr>
</tbody>
</table>

* Mean ± SD of six assays.  
° P < 0.05.

and 24 h later (point A). During the first 24 h of the experiment no change in cholesterol synthesis or protein concentration was noticed. This would indicate that the cells had reached confluency. At point A, the cells were treated with compactin (2 μg/ml), and the incubation was continued for 24 h (until point B), at which time triplicate cultures were used for the measurements. The cholesterol synthesis dropped to a significant degree (upper panel), as indicated at point B. On the other hand, at this point, the cells adhered to the monolayer increased to a maximum (as indicated by the low number of unattached cells, as well as by the high radioactivity associated with the monolayer compared to the respective controls; lower panel). It is known that compactin is a competitive inhibitor of HMG-CoA reductase (19). Suppression of this enzyme by compactin results in the synthesis of large amounts of the enzyme protein as a compensatory response by cells. As expected, when compactin

Fig. 4. Inhibition of cellular cholesterol synthesis by retinoids. Incorporation of [3H]acetate into cholesterol was measured in endothelial cells treated with retinol and retinoic acid (1 μM each) for 24 h. The cells were pulsed with 20 μCi (500 mCi/mmol) of [3H]acetate per flask, and 1 h later, the incorporation into cholesterol was measured. The values are the mean of six measurements; bars, SD.

Fig. 5. Coordinated changes in cholesterol synthesis and cell adhesion: inverse relationship. Endothelial cells were seeded in T-25 flasks as described in "Materials and Methods." They were then treated as follows. O, control; cells grown to confluency in DMEM; A, same as 0 time point; 24 h later. At this point the dishes were switched to medium containing delipidized serum. B, the cells were maintained on medium containing delipidized serum for 96 h prior to the sampling. For cholesterol synthesis, 2 μCi of [4C]acetate (specific activity, 54 mCi/mmol) were added to triplicate cultures, and the cells were harvested 1 h later to measure the incorporation of radioactivity into nonsaponifiable fractions. For cell adhesion, B16 melanoma (F10) cells were labeled with the [4C]-amino acid mixture, and these cells were used for the adhesion assays. At the end of 30 min after cell addition, portions of the medium were withdrawn for counting of the unattached cells. The cell layer was washed twice with DMEM, and the cells were lysed in 0.5% sodium deoxycholate. The lysate was transferred to liquid scintillation vials for the measurement of radioactivity. The values given are the mean of six assays. Bars, SD.
was withdrawn and the measurements were made after subsequent 24 h, there was an overshooting of cholesterol synthesis with a simultaneous decrease in cell adhesion (point C). This change was reversed (point D) on addition of compactin back into the culture. Although the endothelial cultures could be maintained well in delipidized serum-containing medium, prolonged incubation (96 h) resulted in decreased cholesterol synthesis (point E, upper panel). Measurement of cell adhesion showed that at this point there was a slight stimulation in cell adhesion (lower panel, point E).

DISCUSSION

The key findings of this study are: (a) inhibition of cholesterol synthesis in endothelial cells by compactin and by oxygenated sterols facilitates their binding of the tumor cells; (b) endothelial-tumor cell interaction is not influenced by the treatment of endothelial cells with inhibitors of protein glycosylation; (c) dexamethasone and retinol, which cause inhibition of cholesterol synthesis, also promote tumor cell adhesion; and (d) enrichment of lipid depleted cells with cholesterol dispersions inhibits cell adhesion.

It is intriguing that there is a negative correlation between the cellular cholesterol synthesis and the endothelial-tumor cell interaction. Since this interaction is one of the obligatory steps in the multistage metastatic process, it could be considered an in vitro measure of metastasis formation. We have, in this study, used compactin, 25-hydroxycholesterol, and 7-ketocholesterol to inhibit the cellular cholesterol synthesis. Since these agents act at the HMG-CoA reductase step in the formation of mevalonate, it cannot be established unequivocally from this study whether or not non-sterol mevalonate products are also involved in the enhancement of cell adhesion by these agents. However, the finding that cholesterol dispersions inhibit the cell adhesion suggests that, perhaps, it is the reduction of cholesterol that is primarily responsible for the stimulation of cell adhesion. It is also noteworthy that cholesterol is capable of reversing the compactin-induced enhancement in cell adhesion.

The clinical implications of the relation between cellular cholesterol and cell adhesion cannot be ignored in view of the therapeutic use of cholesterol lowering agents in hypercholesterolemia. On the basis of the inhibitory properties of compactin and its analogue mevinolin, these drugs are currently being used against several neoplasms, also causes an increase in plasma cholesterol and induction in HMG-CoA reductase (29).

Enhancement of cell adhesion by dexamethasone is in accordance with several earlier studies on the effects of glucocorticoids in increasing metastases in experimental animals. Many years ago, Zeidman (30) showed that metastases of transplantable Brown Pearce carcinoma are more extensive in cortisone treated rabbits when compared to the controls. Similarly Kodama and Kodama (31) observed that hydrocortisone enhances the hematogenous metastases of Ehrlich ascites tumor in mice. Fidler and Lieber (32) have shown that pulmonary metastases of B16 melanoma were promoted by triamcinolone acetonide. On the other hand, the in vitro effect of dexamethasone on the adhesion of CCRF-CEM or Raji cells to endothelial cells shows the steroid to have an inhibitory effect (33). The enhancement in cell adhesion by dexamethasone observed in our study supports the results from the animal experiments mentioned above. The observation that glucocorticoids inhibit cholesterol synthesis in a wide variety of cells (18) further strengthens this argument.

We have observed that both retinol and retinoic acid stimulated the cell adhesion. Both of these retinoids decreased cholesterol synthesis in endothelial cells. This effect was analogous to that of dexamethasone. Although antineoplastic properties of retinoids are being studied extensively by various workers (as reviewed in Ref. 34), their effects on metastasis formation have not been studied in detail.

Nicolson and Custead (35) have shown that certain chemotherapeutic drugs used in cancer treatment such as bleomycin can cause endothelial damage. Such damage to endothelial integrity can expose tumor cells to the subendothelial matrix and facilitate cell adhesion. We verified such a possibility by examining the endothelial monolayer by scanning electron microscopy (Fig. 2). None of the agents used in our experimental model seemed to cause any morphological changes in the cells as compared to their respective controls (data not given).

The lack of effect of tunicamycin or swainsonine on cell adhesion (Fig. 1) would suggest that oligosaccharide residues may not be involved in this process. Tunicamycin is known to inhibit the dolichol-linked oligosaccharide assembly, while swainsonine inhibits the final trimming of the glycosyl residues in protein glycosylation. If the effect of compactin on cell adhesion was by interference in the phosphodolichol (a mevalonate product) pathway of protein N-glycosylation, one would have expected a similar effect with tunicamycin and swainsonine. This substantiates the importance of cholesterol in regulating the cell adhesion.

It is possible to examine the phenomenon of cell adhesion in light of the model proposed by Liotta and his group. According to this model, laminin present at the basement membrane binds to the laminin receptors located at the tumor cell surface. The possible correlation between the increase in membrane fluidity and the increase in cell adhesion noticed in this study allows one to propose that there exists some mechanism under the tight regulation by membrane fluidity to facilitate the availability of laminin binding sites to the receptors. Since manipulations of the lipid composition of the tumor cells did not affect the binding characteristics (data not given), events that occur on the endothelial surface should be responsible in the regulation of cell adhesion.

ACKNOWLEDGMENTS

We thank Pat Harmon for excellent technical assistance.

REFERENCES

Enhancement in the Adhesion of Tumor Cells to Endothelial Cells by Decreased Cholesterol Synthesis

Chittoor K. Ramachandran, Karen Sanders and George Melnykovych


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/46/5/2520

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/46/5/2520. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.