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Original Article

Role of heparin in the activation and migration of primary human umbilical vein endothelial cells (HUVECs) following signal transduction by CD44 and its ligand hyaluronic acid

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Abstract

BACKGROUND: CD44 is an extracellular matrix molecule that has hyaluronan as one of its principal ligands. The interaction between CD44 and its ligand initiates activation and migration of endothelial cells. Migration of endothelial cells holds importance in neovascularization during cancer metastasis where new blood vessels are formed in order to nurture metastasized cancerous tissue. Studies on bovine endothelial cells showed that greater degree of migrations occurred with addition of lower molecular weight hyaluronic acid fragments (HAF) than the whole molecule of hyaluronan (HA). Our study included addition of heparin to the seedings of HA and HAF at concentrations of 10µg/ml, 100µg/ml, 250µg/ml and 500µg/ml to endothelial cells and measuring the degrees of migrations obtained on migration assay.

METHODS: HUVECs were isolated from umbilical cords obtained from Kasturba Medical College, Manipal, India. The cells were standardized to 2000 cells/ml and cultured in co-star well plates and were seeded with HA and HAF at concentrations of 10µg/ml, 100µg/ml, 250µg/ml and 500µg/ml. Basal level of control were resting cells and PMA treated cells were positive control. Migration assay was done on which the endothelial cells were embedded. After treatments, the migrations were measured and compared. Expression of CD44 was done using SDS-PAGE and Western Blot analysis.

RESULTS: Heparin seems to play a role in the activation and migration of endothelial cells and their sustenance and viability in the medium. In Heparin M199 medium, the endothelial cells showed greater migrations when seeded with HAF than with HA. At 100µg/ml heparinised endothelial cells showed greater degree of migration with HAF than HA added at the same 100µg/ml. In heparin-free medium, the primary HUVECs migrated more at 10µg/ml for cells seeded with HA while they migrated more with HAF at a concentration of 250µg/ml. SDS–PAGE and western blot analysis showed CD44 molecules on the primary HUVECs.

CONCLUSION: The assays were set up and studied in duplicate. Further analysis should be done in order to substantiate the role of heparin in the migration of primary human umbilical vein endothelial cells following signaling with CD44 and HA. This might hold promise for therapeutic research into cancer metastasis and neovascularisation.

KEY WORDS: CD44, hylauronic acid, cancer metastasis, signal transduction, heparin, activation, migration, human umbilical vein endothelial cells HUVECs, neovascularisation.

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CD44 is a ubiquitous transmembrane glycoprotein, cell surface molecule. The most prolific isoform is the CD44 standard that is present in a wide variety of tissues, whereas the other variants of CD44 (CD44v) which are coded for by alternative splicing are much restricted in distribution. Known functions of CD44 are cellular adhesion-aggregation and migration, Hyaluronate degradation, lymphocyte activation, lymph node homing, myelopoiesis, lymphopoeisis, angiogenesis and release of cytokines.[1]

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With respect to the various isoforms and tissue distributions of CD44 molecule, it is but essential that this molecule has various ligands in order to perpetuate the different physiological functions. These ligands include hyaluronate, chondroitin, collagen, laminin and fibronectin.[2] The main ligand being hyaluronate. The amino terminal domain of CD44 is composed of two clusters of positively charged amino acids, which bind a six-sugar sequence of hyaluronate. Hyaluronate is a very long, high molecular weight polysaccharide molecule. It is distributed ubiquitously in the extra cellular space of higher animals. The Hyaluronate molecule has other receptors as well like the RHAMM (receptor for hyaluronic acid mediated motility). The cells' ability to bind to CD44 is finely regulated by the clustering effect of the CD44 molecules by oligomerisation as it is a multivalent interaction. This binding affinity is increased with molecular ionic strength as both molecules are negatively charged and binding is optimal at a neutral pH.[3,4] CD44 adhesions to hyaluronate have been observed to be weak compared to other physiologic adhesions such as those of integrins and cadherins. This appears to be an advantage as the cells need only to transduce chemical signals.[5] This could account for its properties of activation and maturation of lymphocytes.[6]

Neovascularisation or angiogenesis is the process of formation of new blood vessels from pre-existing vasculature that takes place during several biological processes like wound healing and in some pathological conditions like diabetic retinopathy and in tumor growth and metastasis. During these conditions, the endothelial cells sever their association with their neighboring tissue endothelium, migrate and proliferate in the surrounding tissue and re-establish their cell adhesions to the basement membrane and their adjacent cells to form new capillaries.[7]

Both HA and CD44 seem to exert their biological functions through various cell associated receptors and ligands respectively.[8] The most distinct receptors that have been identified so far for HA have been CD44 and RHAMM.[8-10,1] Although many such surface molecules have been implicated for the biological activities of CD44 and HA, the most important has been found to be the interaction involving CD44 with HA that is directly attributed to it activating intracellular signals that is required for the stimulation of the process of angiogenesis.[10,11,1] Several *in vitro* investigations have suggested that these two molecules take active part relevant to HA dependent endothelial functions that are responsible for the process of angiogenesis although this aspect has not been well established *in vivo*.[12-14] Rasmin et al have demonstrated that CD44 molecule is responsible for endothelial cell (EC) adhesion to HA and cell proliferation while RHAMM mediates EC migration through the basement membrane by the use of specific blocking antibodies.

Bovine endothelial cells seemed to show much greater migration when the cells were treated with hyaluronic acid fragments of low molecular weight than when treated with high molecular weight of the hyaluronic acid molecule as a whole. We studied to see the effect in human umbilical vein endothelial cells and also incorporated to see how the endothelial cells would behave when treated with heparin and without heparin.

Materials and methods

Migration assay:

In order to give the endothelial cells a matrix onto which they could attach, gelatinized cover-slips coated with colloidal gold were used. For the preparation of colloidal gold; 5mls of 36.6mM sodium carbonate (B.D.H. Ltd. UK) and 1.8mls of 14.5mM of gold chloride solution (Sigma Chemical company Ltd. UK) were mixed in 11mls of HPLC distilled water. The contents were stirred with a magnetic stirrer and heated to around 95^{o}C. Freshly prepared formaldehyde solution 37% v/v (Sigma Chemical company Ltd. UK) was added to the contents until the colour obtained was blue-black in transmitted light. The colloidal gold solution was added to the gelatinized cover-

slips placed in 8-welled multidishes (Nalgene Nunc International, USA) for forty-five minutes and sterilized in UV light for an hour.

Tissue culture:
The umbilical cords were obtained from the Department of obstetrics and gynaecology preserved in Hank's buffered saline with prior permission and clearance from Kasturba hospital ethical clearance committee. Primary human umbilical vein endothelial cells (HUVECs) were isolated from the cords and added to T75 tissue culture flasks (Corning Costar, USA) with M199 basal medium (Gibco-Brl, UK) incorporated with Penicillin-Streptomycin (Gibco-Brl, UK) and endothelial mitogen (Biogenesis, UK) along with heat-inactivated fetal calf serum (Gibco-Brl, UK). Heparin was also incorporated in the medium (Sigma chemical company, UK), in order to study the comparison between the activation and migration of the cells.

Pre-treatment of primary HUVECs with different concentrations of hyaluronan and hyalorinic acid fragments:
The endothelial cells were passaged to P2 and the cells were standardized to 2000 cells/ml for each pre-treatment. The gelatin-colloidal-gold coated cover slides were placed in the six-welled multidishes (Nalgene Nunc International, USA) and the M199 with or without heparin was incorporated depending on the assay that was set. High molecular weight hyaluronic acid (HA) and low molecular weight hyaluronic acid (HAF) (Lifecore Biomedical, USA) were added in concentrations of 10µl, 100µl, 250µl and 500µl. Resting endothelial cells without any treatment were taken as the basal level of control while PMA at 1µM concentration was taken as the positive control for the migration studies.

Measurement of migrations of the primary HUVECs:
The migrations obtained on the colloidal-gold coated matrix by the effects of the treatments on the endothelial cells were measured using the software, NIH image version 1.62f (NIH, USA). The migrations that appeared as clearing around the endothelial cells on the matrix were traced and given parameters like elongation, length, breadth, area, perimeter etc. using the software and the data obtained were thrown into graphs using Prism graphpad application. Around 70 migrations per slide for each assay that was set up with different concentrations of HA and HAF were traced and studied.

SDS-PAGE and western blot:
Prior to running the concentrated protein samples on the gel, the gel apparatus(Atto, Japan) was set up accordingly with the various compartments in place. The resolving gel (10%) was prepared by adding 8.04 mls distilled water, 6.66 mls of 30% Acrylamide/bis-Acrylamide, 5mls of 1.5 M Tris-HCL(8.8), 200ul of 10% w/v SDS, 100ul of 10% w/v APS and 10ul TEMED (Sigma chemicals company Ltd. UK). The stacking gel was prepared by adding: 3.05 mls dist water, 0.65 mls Acrylamide/bis-Acrylamide, 1.25 mls of 0.5M Tris-HCL(pH 6.8), 50ul of 10% w/v SDS, 25ul of 10% w/v SDS, 25ul of 10% w/v APS and 5ul of TEMED (Sigma chemicals company, Ltd. UK) in that order. Samples were electrophoresed for approximately 1.5 hours at 100V/20amp per gel. The protein samples were then transferred onto PVDF membranes by Western blotting. The blotted membrane was washed in PBS containing 0.1% v/v Tween-20 in order to prevent non-specific binding by the antibodies. The primary antibodies added was Bric 238 (New England Biolabs, UK) to which the secondary antibodies labeled with Horseraddishperoxidase (New England Biolabs, UK) was added with anti-Bric 238 being goat anti-mouse secondary antibody. Detection of antigen was carried out by the HRP (home-made) detection kit. This was prepared by adding 12mls of 100mM Tris-HCl, pH 8.5, 3.8ul of 30% hydrogen peroxide, 60ul of 250mM luminol (diluted in 100mM tris-HCl (pH8.5) and 26.6ul of 90mM p-Coumaric acid (diluted in DMSO).

Results

With HA treated cells in heparin M199 medium, migrations of primary HUVECs were more at 10 µg/ml but not relatively greater when compared to the PMA treated cells but were quite greater to those of the resting cells. In heparin-free medium, HA treated cells migrated more at 250µg/ml but with the same degree of comparison to the previous when compared to the PMA treated cells. The migration assay of primary HUVECs treated with low molecular weight hyaluronic acid fragments at 100µg/ml showed much greater migrations with typical directional migrations when incorporated with heparin in the M199 medium than when compared to the PMA treated control itself (Figure 1). The same was not observed for HAF treated HUVECs in heparin-free M199 medium, where greater degree of migrations were seen in cells seeded at10µg/ml which was slightly greater when compared to the resting cells but not so when compared to the positive control.

CD44 expression analysis for the assays were done by SDS-Page that detected bands at 37KDa (Figure 2a) and matched with that of the molecular weight marker for CD44. The antigens of CD44 were detected using primary and secondary anti Bric 238 antibodies using Western Blot analysis (Figure 2b).

Discussion and conclusions:

The slides coated with colloidal-gold seemed to show better and discrete migration patterns of endothelial cells compared to the slides that were fed with heparin-free M199 and they maintained good cell morphology when seeded at 2000cells/ml. According to Sneath et al, this phenomenon could be explained by the fact that glycosylation of exon v3 of CD44 molecule with heparan sulphate enables it to bind more efficiently to heparin binding growth factors and cytokines including fibroblast growth factor b (bFGF) and heparin binding epidermal growth factor (HB-EGF). This binding acts as a reservoir for the growth factors and cytokines.

The cellular and discrete patterns of migrations of endothelial cells in heparin medium (Refer Figure 3) maybe due to the possession of exon v10 of CD44 of a serine-glycine motif that acts as a chondroitin sulphate attachment and v10 can also promote homotypic or heterotypic cell-cell adhesion that may explain the stronger attachment of endothelial cells to the gelatin-colloidal gold coated slides. This heterotypic tight adhesion of the cells might provide the basis for the assay showing better results in heparin medium, as the cells have the facility of the preferential adherence motifs exposed to the treatments of HA/HAF and help in trapping the essential growth factors for the survival and health of the cells.

From the study, heparin seems to play a certain role in the activation and migration of the endothelial cells. Primary HUVECs showed a greater degree of migration in heparinized medium for HAF at 100µg/ml compared to HA in heparinized medium while in heparin-free medium, cells showed more migration and activation with HA than with HAF but when compared to the positive control in both sets of assays they were much less in their degree of migrations. This seems to hold good that the low molecular weight fragments of HAF are more efficient in causing migration of endothelial cells as was reported in the case of bovine endothelial cells.[15]

The interactions of the endothelial cells with the extra cellular matrix through their mediators like HA and CD44 are integral for the process of neovascularisation.[16-18] Heparin seems to influence the endothelial cells in their activation and migration, especially in the case of low molecular weight HA fragments. Heparin may play a role in the migration due to the increased chemotaxis and chemokinesis of the endothelial cell growth factor (ECGF) as was studied by Terranova et al using a modified boyden chamber and heparin was shown to potentiate the chemotactic activity of ECGF. This particular phenomenon may play a relevant role in the study for potential targets for tumor metastasis and angiogenesis.[19]

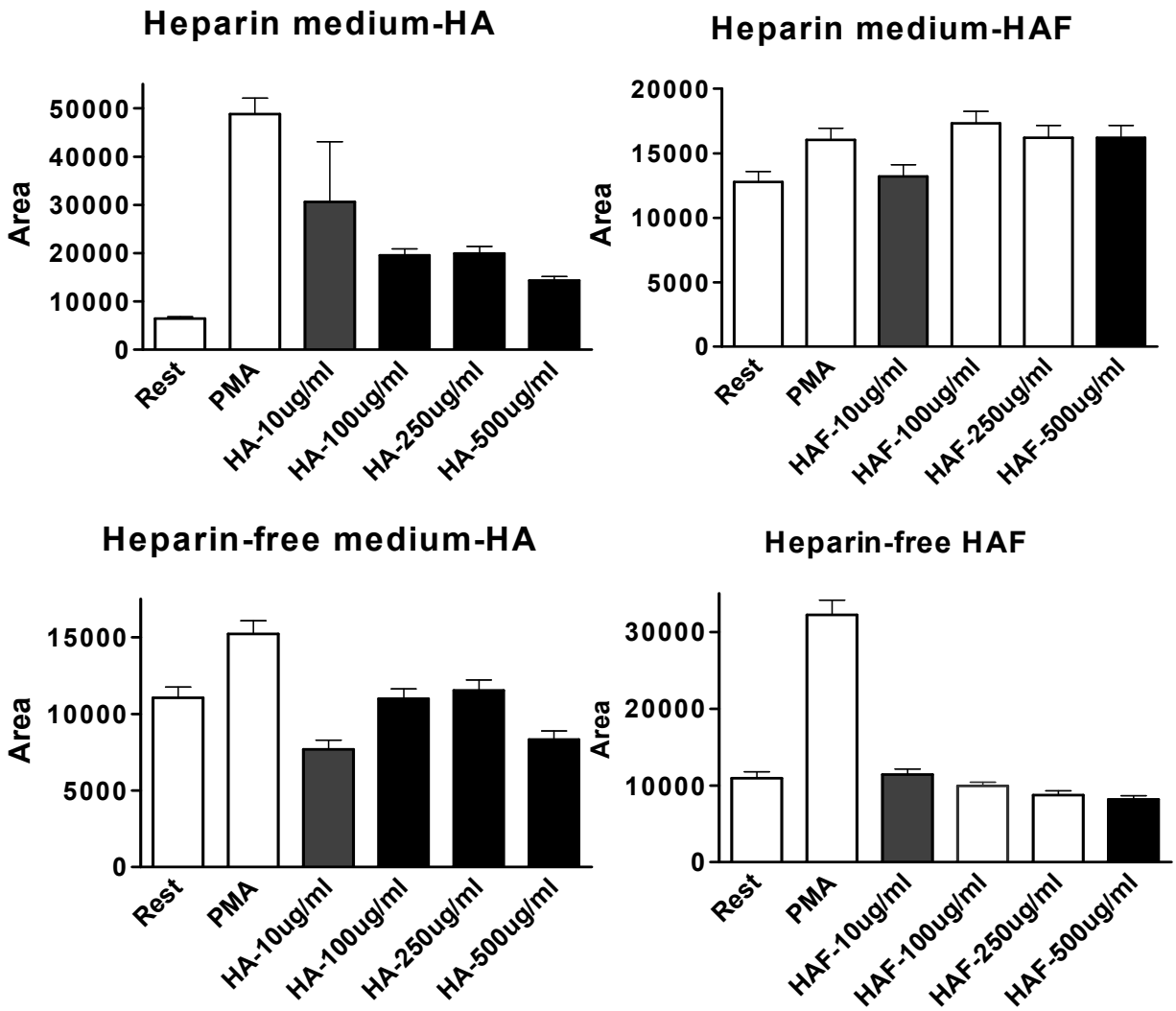

Figure 1: Area of migrations (y-axis) of the HUVECs with respect to their treatments of HA and HAF at concentrations listed on the X-axis in response to heparin and without heparin respectively.

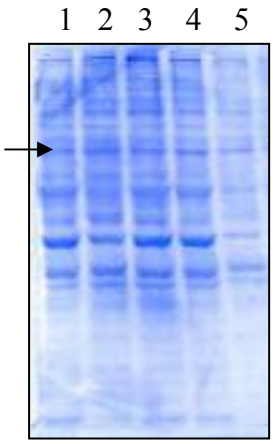

Figure 2a: SDS-Page showing CD44 expression in treatments of HUVECs. Lane 1to lane 5 loaded were the molecular weight markers, the resting cells, PMA treated cells, HA and HAF respectively. Arrow shows CD44 at 37Kda.

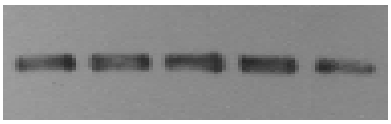

Figure 2b: Western Blot detecting antigens of CD44 treated with primary Bric 238 and secondary antibody labeled with HRP was goat anti-mouse anti-Bric 238.

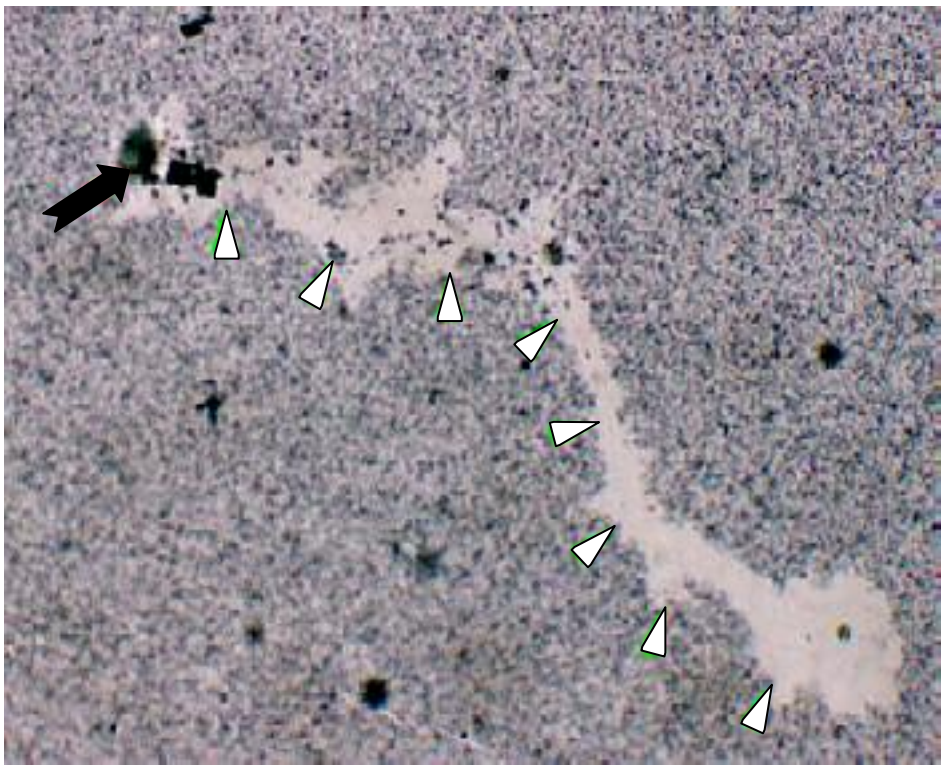

Figure 3: shows the HUVEC and its migratory track (white arrows) along the gelatin-colloidal gold coated cover-slides that act as a matrix for the HUVECs to adhere to and form discrete tracks while they migrate. The black arrows show the position of the endothelial cell relative to the migratory tracks. The up-down arrow shows the path of migration left by the endothelial cell. Magnification at 10X.

There are several other ligands for CD44 and HA. RHAMM has been identified as one of the ligands for HA and heparin has been found to bind to RHAMM and it might mediate the process of angiogenesis through activational migration of the endothelial cells.[20]

Further studies may target the interactions of the high molecular and low molecular weight HA with respect to heparin and the ligands. Investigations that are more specific to the respective receptors with the help of blocking antibodies to non-specific molecules are recomended. Assays should be set up in duplicate or triplicate in order to obtain authentic results. But this study and the assays conducted showed that there seems to be some correlation to the activation and migration of primary HUVECs in heparin, and with response to the low molecular weight fragments of HA.

This might hold therapeutic significance to curtail the process of neovascualrisation and angiogenesis during cancer metastasis. Tumors that are deprived of their oxygen supply cannot grow more than 1-2mm in diameter, after which they are necrotized. Tumors, in order to survive and progress, need a rich supply of blood; to fulfill this purpose there is induction of blood vasculature that form small capillaries around the growing tumor and nourish it with

oxygen and nutrients. This process of revascularization is called 'angiogenesis'.[21,22] Neoplasms are able to model small capillaries to suit their microenvironment due to the action of vascular endothelial cell growth factor (VEGF) and angiopoietins (APNs) that are stimulated by hypoxia inducing the laying down of immature vessel walls which are later remodeled with the help of APNs. Tumors often secrete excess of VEGF that causes vessel leakage and edema. This is one area where extensive research has been carried out to thwart the supply of VEGF through monoclonal antibodies directed against receptors of VEGF. The growth and survival of a tumor rests on the abundant vasculature surrounding it that nourishes the neoplasm with oxygen and nutrients. Oxygen can diffuse radially from capillaries for 510-200µm. If the tumor exceeds this distance, apoptosis of the tumor cells takes place. This goes to show that tumor cells and metastasis are directly proportional to the process of angiogenesis.[23,24,25] Through the study of heparin and its effect, the inherent pathways involved for the migrations could be elucidated further and a concerted effort to synergistically inhibit the migrations of the endothelial cells could be instituted as a therapeutic modality.

It is not only the above pathways that a tumor can adopt to flourish in its microenviornement but there are a lack of endogenous factors like IFN-β, which is an anti-angiogenic factor, that inhibits IL-8 and basic fibroblast growth factor (bfgf). Therefore, cytokine targeted monoclonal antibody therapy alone might not be able to thwart cancerous neoplasms, but there should be institution of a cocktail that will help inhibit the progression of tumors through various pathways.

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