

RESISTIN AND HIGH GLUCOSE CONCENTRATIONS-ACTIVATION OF HUMAN SMOOTH MUSCLE CELLS INDUCES ENHANCED MONOCYTE CHEMOTAXIS

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Abstract

Objectives. Recent data indicate that upon activation by resistin and high glucose concentrations (HG) vascular smooth muscle cells (SMC) acquire pro-inflammatory properties. We questioned whether resistin and HG-activated SMC generate an enhanced monocytes chemotaxis and if the chemokine fractalkine (Fk) is involved in the process. **Material and Methods:** SMC were incubated with resistin or/and HG and the conditioned medium was used for monocytes chemotaxis assays. The role of Fk was assessed by blocking the Fk receptor, CX3CR1, on monocytes (U937 cell lines) prior to the chemotaxis assay. The quantification of migrated monocytes was assayed under an inverted microscope and statistically analyzed. **Results:** (i) conditioned medium (CM) collected from SMC incubated with resistin in the presence or absence of HG triggered a significant increase (25–100%) of monocytes chemotaxis as compared to controls; (ii) blocking the CX3CR1 receptor significantly decreased the monocyte chemotaxis towards resistin-treated SMC. **Conclusions:** Resistin±HG increases the expression of chemotaxis inducers in human SMC and the ensuing monocytes chemotaxis by a mechanism in which Fk plays a major role.

key words: resistin, high glucose concentrations, smooth muscle cells, monocytes, chemotaxis, fractalkine, fractalkine receptor.

Background

Resistin was originally described as an adipocyte-secreted peptide that induces insulin resistance in rodents [1]. Recently, resistin emerged as a significant local and systemic regulatory cytokine implicated in vascular wall inflammation and cardiovascular disease progression [2, 3, 4]. Accumulated data

indicate that resistin accelerates atherosclerosis by aggravating the inflammatory conditions in the vessel wall through stimulation of monocyte infiltration and activation of endothelial cells (EC) and vascular smooth muscle cells [2].

Fractalkine, also known as CX3CL1, is the unique member of the fourth class of chemokines and mediates both chemotaxis

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and adhesion of inflammatory cells via its highly selective receptor CX3CR1. Recent data implicate Fk and its specific receptor CX3CR1 in the directional migration of various cells to atherosclerotic sites [5, 6, 7, 8].

In humans, resistin and HG are concomitantly present at elevated concentration in diabetic patient's plasma and both are pro-inflammatory agents acting on vascular cells [9, 10]. We have recently reported that resistin and HG induces human EC dysfunction by increasing the expression of endothelial adhesion molecules, fractalkine and P-selectin and the ensuing monocytes adhesion, key events in atherosclerosis and in diabetes [11]. We have also recently shown that HG induces an up-regulation of Fk in SMC and that this chemokine is involved in monocytes adhesion to SMC and in monocytes-SMC cross-talk, by increasing the expression of pro-inflammatory mediators in both cells [12, 13].

There are no data on the effect of R and HG on SMC activation and on the subsequent monocytes infiltration.

Since high resistin and HG induce independently SMC activation, we questioned whether when acting together, the monocytes chemotaxis toward SMC is enhanced and if the axis CX3CL1/CX3CR1 is involved in the process.

Materials and methods

Cell culture

Human aortic smooth muscle cells (SMC) were isolated from the media of fetal thoracic aorta and characterized as a pure cell line devoid of any contaminants. The cells exhibited an elongated spindle shape morphology (as assessed by phase-contrast microscopy), exhibited bundles of cytoplasmic

myofilaments and numerous caveolae at the cell periphery (as demonstrated by electron microscopy), and were positive for smooth muscle alpha-actin, and for vinculin, and negative for von Willebrand factor. SMC were cultured as described [13].

Monocyte-like cell line U937 (kindly donated by Prof. S.C. Silverstein, Columbia University, NY, USA) were grown in suspension in the RPMI 1640 culture medium containing 5% FCS and were split 1:5, twice a week.

Materials

Dulbecco's modified Eagle medium (DMEM), RPMI-1640, antibiotics, D-glucose, 8-bromo-cAMP, FMLP (N-Formyl-Methionyl-Leucyl-Phenylalanine), and all other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany); fetal calf serum (FCS) was from Gibco-Life Technologies, Medist SA (Bucharest, Romania); Human resistin and anti-CX3CR1 antibody were purchased from Santa Cruz and the Boyden chambers with 5- μ m pore size polycarbonate filters from Corning Incorporated (Corning, NY, USA).

Experimental design

The U937 cells were incubated with cAMP (1mmol/l), in order to differentiate them to monocytes or activated by R (100ng/ml) for 24 or 48 hours. SMC were incubated with R (100ng/ml) and/or HG (25mM), and after 24 hours the conditioned medium was collected and used for the chemotaxis assay using Boyden chambers with 5 μ M-diameter filters. To evaluate whether Fk is specifically involved in monocytes chemotaxis, the CX3CR1 expressed on monocytes was blocked with an

anti-CX3CR1 antibody (5µg/ml) for 1 hour, prior to the chemotaxis assay.

The monocytes migrated to the lower compartment of the Boyden chambers were counted under an inverted microscope.

Monocyte chemotaxis assay

Chemotaxis was assayed in Boyden chambers containing permeable 5-µm pore size polycarbonate membranes (Corning Incorporated, Corning, NY, USA). Monocytes were cultured at 37°C with 5% CO₂ in DMEM culture medium supplemented with 10% FCS and penicillin G (100 IU/ml), streptomycin (100 µg/ml) and gentamicin (50µg/ml) and incubated with cAMP, or R for 24 or 48 hours. The supernatant from cultured SMC unstimulated and stimulated with R and/or HG (25mM) for 24 h were collected, diluted in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and placed in the bottom wells of the Boyden chambers (600µl per well). Polycarbonate membranes were then fixed in place in 24-well plates, to separate the bottom from top compartments and 5×10⁵ monocytes in 200µl PBS containing 0.1% BSA were added to the upper wells. The loaded chambers were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 180 min. Monocyte chemotaxis in various conditions was quantified by counting (by microscopy using a hemocytometer) the cells that traversed from the upper to the bottom well of the Boyden chambers.

Statistical Analysis

The data collected were expressed as the means±standard deviation (SE). Normally distributed data were analyzed using a paired *t-test* or analysis of variance (ANOVA). Non parametric data were analyzed by the Kruskal-

Wallis and Mann-Whitney statistics. Linear regression analysis was performed to determine the degree of correlation between variables. The conservative, non-parametric Sperman statistic (*p*) was used. Significant differences were accepted when two-tailed analyses yielded *P* < 0.05.

Results

Chemotaxis of monocytes

In preliminary experiments we tested U 937 cells ability to perform chemotaxis toward activated SMC. Our results revealed that U 937 cells had a low chemotactic activity towards the conditioned medium (CM) collected from SMC activated by R, HG or R and HG, while incubation of U 937 cells with cAMP(1mmol/l) for 48 hours or resistin (100ng/ml) for 24 hours resulted in an increased chemotaxis towards activated SMC ([Figure 1](#)). Since we wanted to explore R effects in the process, in the following experiments we have used resistin-activated U 937 cells.

Effect of resistin and high glucose activation of human SMC on monocytes chemotaxis

To evaluate if resistin and/or high glucose induces a pro-inflammatory phenotype in SMC leading to an increased monocyte chemotaxis, the monocytes migration towards the CM of SMC treated with R, HG or resistin and high glucose (HGR) was quantified. We found that the CM collected from SMC incubated for 24h with resistin significantly increased the monocyte chemotaxis (25 % increase over the control level), compared with CM from untreated SMC. Similarly, HG-treated SMC conditioned medium generated an increased monocytes chemotaxis at a

comparable level to resistin-activated SMC (45 % increase over the control level) while the conditioned medium from SMC treated with

HGR induced a 100% monocytes chemotaxis, indicating that resistin and high glucose have a synergic effect in the process (Figure 2).

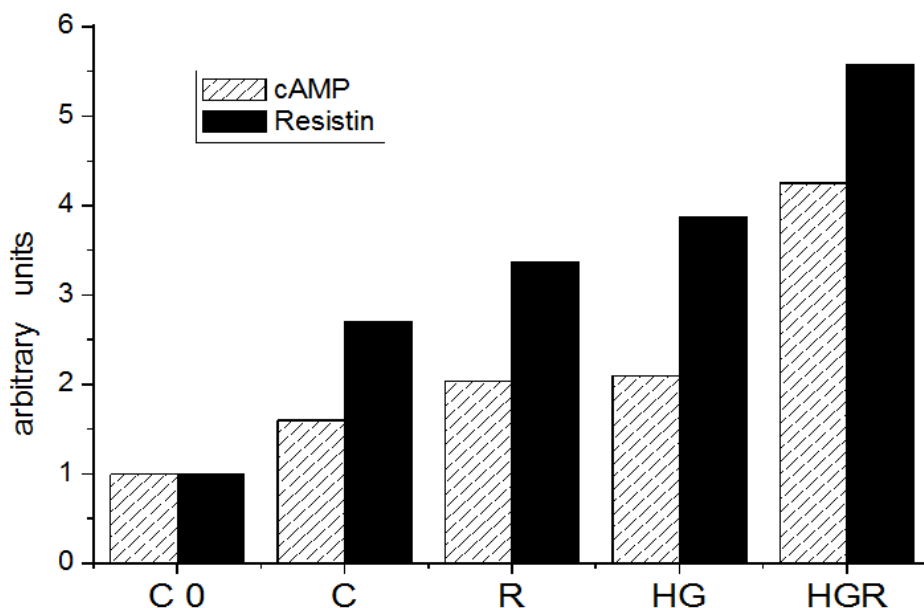


Figure 1. Chemotaxis of cAMP or resistin activated-monocytes to the conditioned medium from human SMC activated with resistin (R), high glucose concentrations (HG) or resistin and high glucose concentrations (HGR). cAMP or resistin activation of U 937 cells increased their chemotaxis towards conditioned medium from inactivated SMC (C) or activated SMC for 24h with R, HG, and HGR, compared to non-activated U937 cells (C 0). The migrated monocytes were counted using a hemocytometer and normalized over monocytes migration to DMEM culture medium. The chemotaxis assay was performed in Boyden chambers for 3h. Data are expressed as means of three experiments.

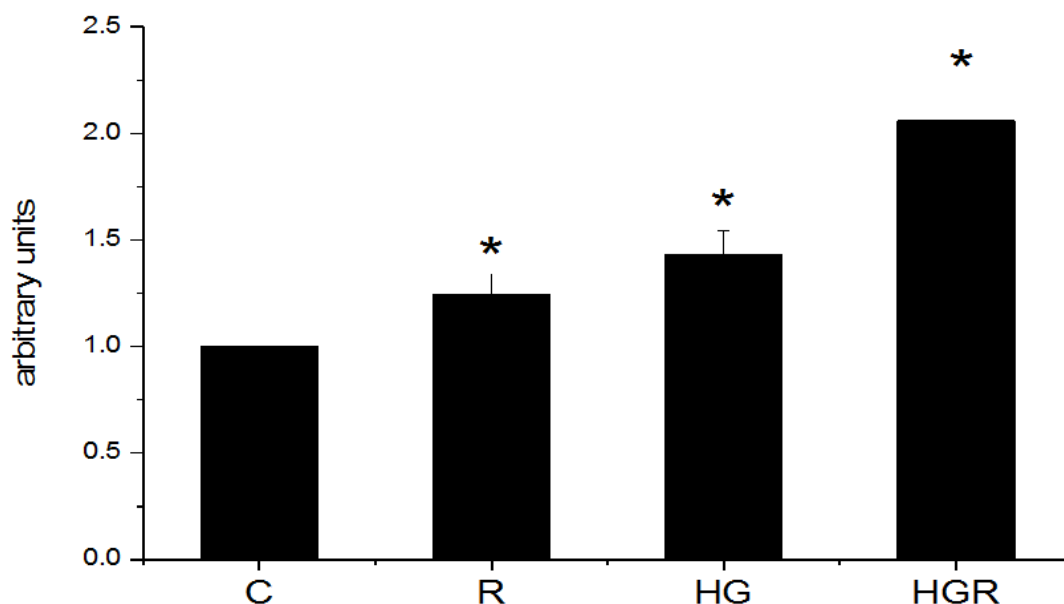


Figure 2. Migration of resistin activated monocytes towards the condition media collected from non-activated human SMC (C) and SMC activated for 24h with resistin (R), high glucose 25mM (HG), or resistin and HG (HGR). Chemotaxis was assayed in Boyden chambers for 3h. The results are expressed as fold induction over the control level (C), considered as 1. *Significantly different from control $p < 0.05$, $n = 3$.

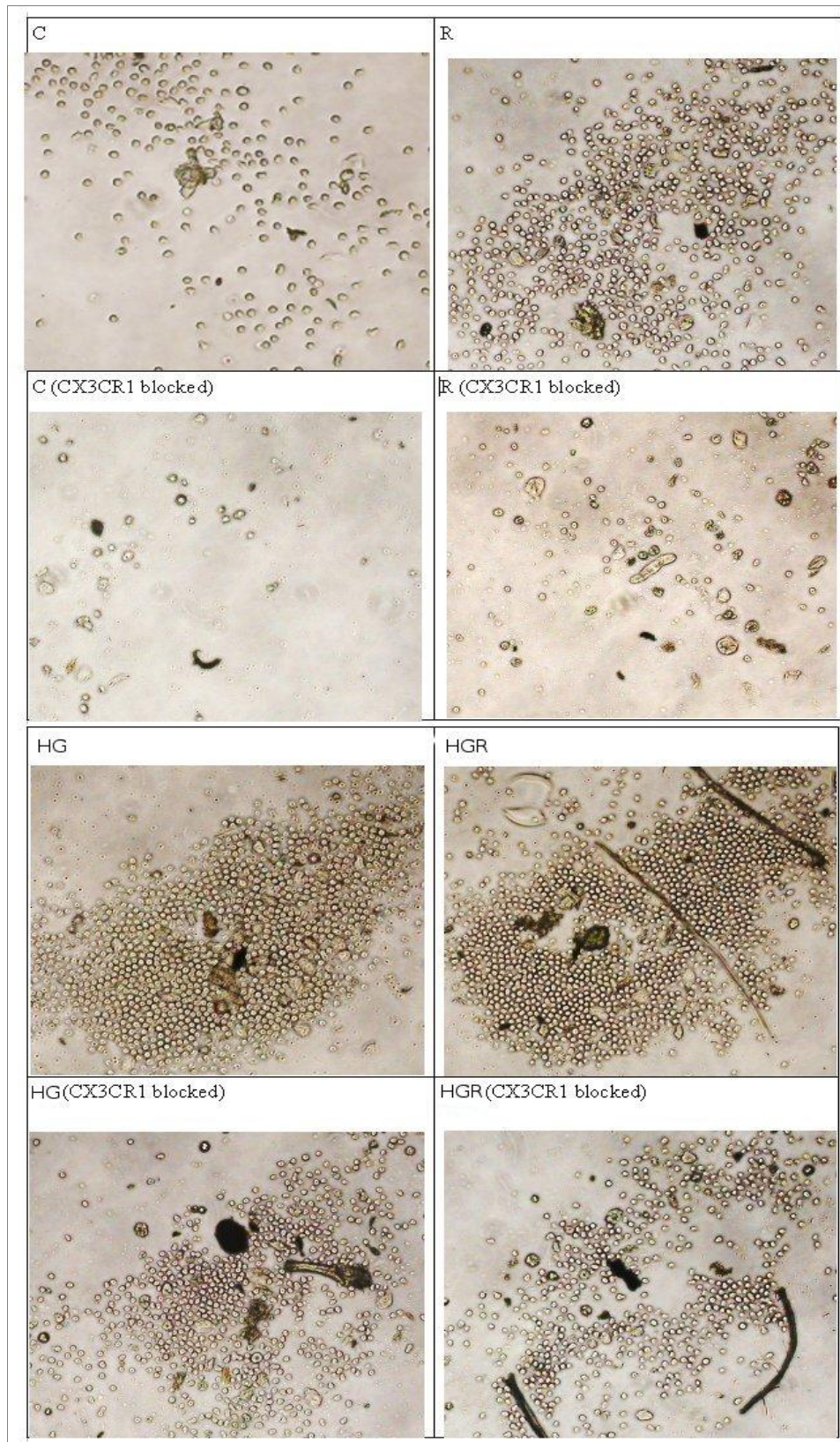


Figure 3. Optical microscopic images of monocytes chemotaxis towards the conditioned medium of SMC treated with resistin (R), high glucose (HG) or resistin and HG (HGR). Blocking the fractalkine receptor by a specific anti CX3CR1 antibody reduces monocytes chemotaxis to condition media collected from activated SMC (CX3CR1 blocked). Monocytes migrated to the bottom wells of the Boyden chambers were visualized using an Olympus microscope (x100).

Mechanisms involved in monocytes chemotaxis towards the factors secreted in the condition media by SMC activated with resistin±HG; role of fractalkine receptor

Since in previous studies we have shown that HG upregulated fractalkine expression in SMC [13], we questioned if Fk is implicated in the monocytes chemotaxis towards the factors secreted by SMC activated by HG±resistin. To this purpose, we performed chemotaxis assays employing monocytes preincubated with specific antibody to block CX3CR1 prior to assay. Optical microscopic and statistical analysis revealed that blocking the CX3CR1 on monocytes, reduced their

chemotaxis to HG and resistin±HG treated SMC (Figure 3). Moreover, the chemotaxis assays showed that when the conditioned medium from SMCs activated by resistin was used as chemoattractant, the number of migrated monocytes with CX3CR1 blocked was significantly lower, compared with the number of migrated monocytes with a functional fractalkine receptor (Figure 4), indicating that Fk may be involved in monocytes migration towards activated SMC.

Monocytes migration towards the conditioned medium of high glucose ± resistin activated SMC was also decreased when the Fk receptor was blocked on monocytes but not a statistically significant level (Figure 4).

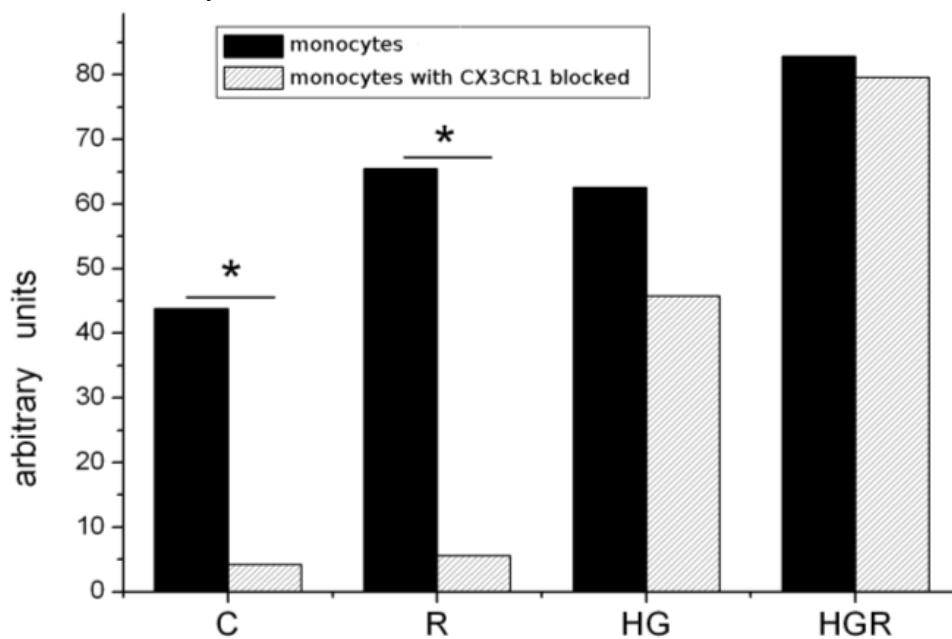


Figure 4. Quantification of resistin activated monocyte chemotaxis towards the CM collected from SMC treated with resistin (R), high glucose (HG) or resistin and HG (HGR). Prior to chemotaxis assay, monocytes were activated for 24h with resistin and then incubated for 1h with anti CX3CR1 antibody. Note that blocking the fractalkine receptor reduces monocytes chemotaxis toward the CM of resistin treated SMC and had no significant effect on monocytes chemotaxis to HG or HGR–treated SMC. Migrated monocytes were counted using a hemocytometer and normalized over monocytes migration to simple DMEM medium. *Significantly different from control. p<0.05, n=3.

Discussions

Monocyte/macrophage infiltration in the vessel wall is a key event in atherosclerosis and in accelerated atherosclerosis associated

with diabetes. Cytokines and chemokines have leading roles in this process [14, 15]. We have recently shown that resistin and high glucose have pro-inflammatory effects in human endothelial cells by increasing the expression

of the cell adhesion molecules (P-selectin and fractalkine) and the consequent monocytes adhesion [11]. We have also shown that in high glucose conditions, human SMC display a proinflammatory phenotype by up-regulating the expression of the cell adhesion molecules and chemokines (fractalkine and MCP-1) and by increasing monocytes-SMC adhesion [16]. To further understand the effects of R and HG conditions on vascular wall inflammation, we searched on the effects of both inductors on monocytes chemotaxis towards activated smooth muscle cells and the factors they secrete. Our data showed that monocytes chemotaxis is increased towards SMC activated by resistin or HG conditions; moreover, in conditions of activating SMC by both R and HG, their effects were synergic. To understand the mechanisms involved and considering that Fk is involved in monocytes adhesion to HG activated SMC, we searched for the role of Fk/Fk receptor in monocytes chemotaxis. Our data revealed that blocking Fk receptor on monocytes abolished completely monocytes transmigration toward resistin-activated SMC, indicating that Fk/Cx3CR1 axis is mainly involved in the process. In contrast, in conditions of activating SMC with HG or HGR, blocking FK receptor had no statistically significant effect on monocytes chemotaxis, suggesting that other chemokines may have leading roles in the process. Experiments to uncover the chemokines induced by resistin and/or HG in human smooth muscle cells are in progress and will help to clarify their role in monocytes chemotaxis.

Conclusion

Factors released by activated SMC enhance monocytes chemotaxis in general and of activated monocytes in particular. The data suggest that in the vessel wall, high concentrations of resistin and glucose present in diabetic conditions increase the expression and secretion of chemokines by human SMC, which in turn enhance monocytes migration into the affected vessels. Moreover, Fk is specifically involved in resistin-induced monocytes chemotaxis. In conditions of resistin-, and high glucose – activated SMC, monocytes chemotaxis is further increased, suggesting that together, the inductors may up-regulate in SMC the expression of different chemokines leading to an increase in monocytes accumulation in the inflamed vessel wall with possible consequences in vascular diseases evolution. Our data add to the understanding of the mechanisms involved in monocytes accumulation and retention in the inflamed vessel wall and indicate new targets for anti-inflammatory therapies in diabetes and atherosclerosis.

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