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CALCITONIN EXPRESSION OF HIGH ENDOTHELIAL VENULES DURING LYMPHOCYTE MIGRATION IN HUMAN PHARYNGEAL TONSIL

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ABSTRACT

The migration routes of lymphocytes through high endothelial venules (HEVs) of control and hypertrophic pharyngeal tonsil (HPT) tissue sections were investigated by immunohistochemistry using the expression of a hormone [calcitonin (CT)] and two calcium-dependent endothelial adhesion molecules (E-selectin and P-selectin), as well as electron microscopy. A marked increase in CT-specific staining was observed in the endothelial cells of HEV in the HPT group compared to the control group. Expressions of E-selectin and P-selectin on HEVs of control group were faint, when compared to the strong expression of these selectins on HEVs of HPT. Electron microscopically, we demonstrated that lymphocytes transmigrated through HEV and observed the close membranous contact between endothelial cells and lymphocytes during this process. We speculate that increasing CT during inflammation may be important for lymphocyte migration through the HEVs via controlling the expression of E–selectin and P-selectin.

Keywords: lymphocyte migration, human tonsil, high endothelial venule, calcitonin, E-selectin, P-selectin

High endothelial venules (HEVs) are specialized vessels that support lymphocyte migration from peripheral blood into secondary lymphoid organs. The migration of lymphocytes required for specific immune reactions is a multi-step process, resulting in rolling, adhesion, activation and migration of lymphocytes (1).

Lymphocyte migration from the circulation into the stroma of a lymph node involves a multistep sequence of interaction between lymphocytes and endothelial cells of HEVs (2-4). It is known that adhesion molecules play an important role for lymphocyte migration through endothelial cells (5) but the mechanisms of the process are not completely understood. This process includes initial low-affinity interaction mediated by selectins followed by chemokine-mediated upregulation of lymphocyte integrin affinity, and then integrin-mediated firm adhesion of the lymphocyte to HEV (6). There are two major classes of cell adhesion molecules; the first is calcium-independent including immunoglobulin superfamily and integrins, and the other is calcium-dependent consisting of cadherins and selectins (7).

Selectins are a family of adhesion molecules that mediate the initial adhesive step, characterized by leukocyte capture and

rolling on the endothelial surface under vascular shear flow. Three types of selectins are known; L-selectin is expressed on most leukocytes, while E- and P-selectin are expressed on activated vascular endothelium. Selectin-mediated cell-cell interaction is a prerequisite for subsequent firm attachment and transmigration of leukocytes (7-9). As a result, lymphocytes bind firmly to endothelium and migrate out of the vessel. Selective migration is thus critically controlled by the expression of selectins and their ligands as well as other molecules mediating the adhesion cascade during migration (10). However, Perry et al reported that the expression of P- and E-selectin on HEV changed during infections, and requirement for E-selectin was low in routine re-circulation of lymphocytes through the adenoid (11). In this study, E- and P-selectins expressed on endothelial cell will be discussed in the context of lymphocyte transmigration through HEV.

CT, secreted by the parafollicular cells of the thyroid, is a peptide present in many tissues, like lungs, liver, intestine, central nervous system and uterus (12-14); it diminishes the serum calcium concentrations by inhibiting osteoclastic activity and increases the cell calcium level (15). The mechanisms of action of CT as a paracrine or autocrine effector on some biologic process like embryo implantation are known (16), but its role in migration of lymphocytes in the pharyngeal tonsil is not completely understood.

Although there are several reports on expression of adhesion molecules from HEVs, no reports are available on the regulation of these molecules. This study is perhaps the first one describing the CT expression of HEV in human pharyngeal tonsil. We postulate that the CT-induced up-regulation of E-selectin and P-selectin expression of HEV results in the adhesion of lymphocytes to endothelial cells of HEV, thereby facilitating migration of the lymphocytes.

MATERIALS AND METHODS

Patients and Specimens

Specimens of pharyngeal tonsils were obtained from 28 children aged between 3 to 12 years, during adenoidectomy operations. Pharyngeal tonsils were obtained from 10 age-matched control cases that underwent bronchoscopy for foreign bodies in the airways. Adenoids were considered normal if there was no history of upper airway and/or middle ear infections during the previous two months. In each of the control subjects, informed consent was obtained from the parents to carry out biopsies. Each tissue fragment was divided into two parts, and each part was processed with different procedures.

Light Microscopy

One of the tissue fragments was fixed in neutral formalin (10%), dehydrated in graded series of ethanol, and embedded in paraffin. Five micron thick serial sections were cut and slides were immunostained with anti-E selectin (1:100; NCL-CD62E-382, Neomarkers), anti-P selectin (1:100; NCL-CD62P-367, Neomarkers) and anti-CT monoclonal antibody (mAb) (1:100; RB-1519P, Neomarkers) by utilizing a streptavidin-Biotin technique (DAKO).

All of the immunostained sections were reviewed by two blinded histologists. Slides were examined under low power (X4 objective) to identify regions containing anti-E-selectin, anti-P-selectin and anti-CT mAb staining endothelial cells. Five randomly selected areas were scored, and in sections where all of the staining appeared intense, one random field was selected. The proportion of stained cells in each selected field was determined by counting at high magnification. At least 100 endothelial cells were scored per X 40 field for each group. All sections were scored in a semiquantitative fashion, considering both the intensity and percentage of cells staining at each intensity. Intensities were classified as 0 (no staining), +1 (weak

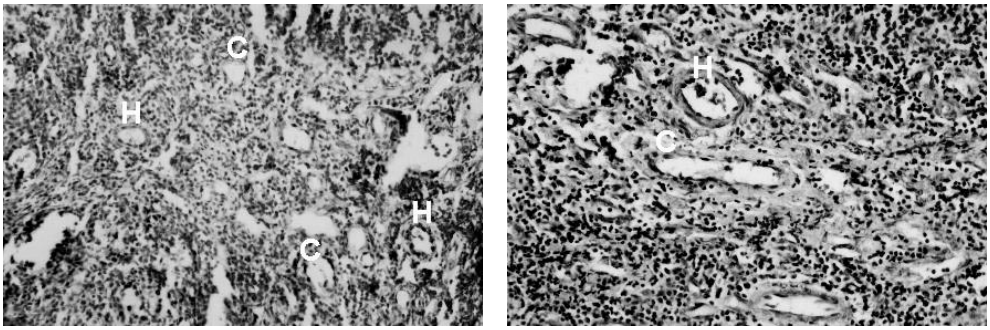

Fig. 1. E-selectin: Immunostaining of the control group (left) compared to the hypertrophic group (right) demonstrates a significant increase (p=0.001) in the number of high endothelial venules (H) showing intense staining. Low endothelial venules (C) show very weak staining in both groups. Original magnification X100 (left) and X400 (right).

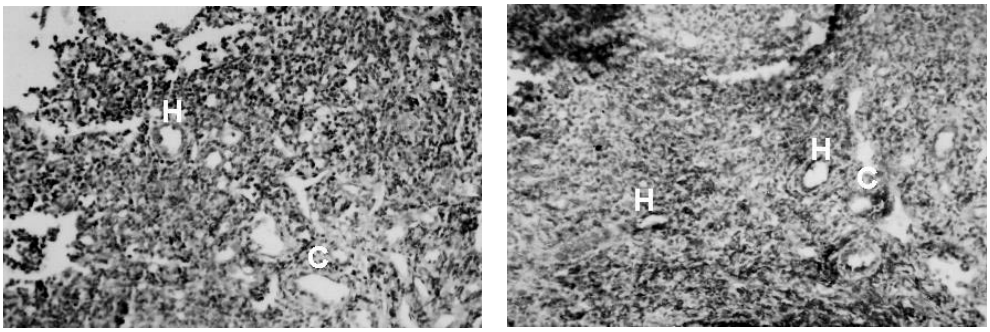

Fig. 2. P-selectin: Immunostaining of the control group (left) compared to the hypertrophic group (right) demonstrates a significant increase (p=0.001) in the number of high endothelial venules (H) showing intense staining. Low endothelial venules (C) show very weak staining in both groups. Original magnification X100.

staining), +2 (distinct staining), and +3 (very strong staining), and 10% groupings were used for the percentage of cells that stained positive. For each slide, a value designated HSCORE was obtained by application of the following algorithm: HSCORE = $\sum$ Pi (i+1), where i = intensity of staining with a value of 1, 2 or 3 (weak, moderate, or strong, respectively) and Pi being the percentage of stained endothelial cells for each intensity, varying from 0% to 100%.

Data were expressed as mean ± standard deviations (SD), or means with 95% confidence interval (CI), where appropriate. Differences in outcome measures between the groups thus generated were examined by analysis of variance (ANOVA) with Duncan's significant difference test for post hoc comparisons and Bonferroni corrections. A p value of <0.05 was considered statistically significant. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) Version 10.0 for Windows (SPSS Inc, USA).

Electron Microscopy

A second tissue piece from each subject was fixed in cold 4% glutaraldehyde in 0.1m cacodylate buffer (pH 7.4) for 4 hours at +4ºC and postfixed in 1% osmium tetroxide in 0.1m phosphate buffer (pH 7.4) for 2 hours at +4ºC. The specimens were then dehydrated in a graded ethanol, passed though propylene oxide, and embedded in araldite. The

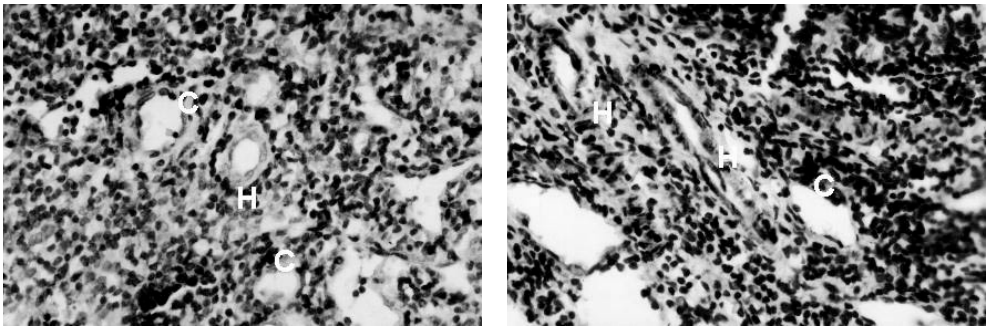

Fig. 3. Calcitonin: Immunostaining of the control group (left) compared to the hypertrophic group (right) demonstrates a significant increase (p=0.002) in the high endothelial venules (H) showing intense staining. Low endothelial venules show (C) very weak staining with calcitonin in both groups. Original magnification X400.

compartments of the pharyngeal tonsils were determined by light microscopy on the semi-thin sections. Ultrathin sections of these areas were then carried out by a Reichert OMU ultramicrotome and stained with uranyl acetate and lead citrate. Observations were made with ZEISS EM 900 electron microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

The distribution of the adhesion molecules within the control pharyngeal tonsil were examined with immunohistochemistry on serial sections and were compared with those in HPT. A striking stain of E-selectin was detected on HEV in both groups, but the intensity was higher in the HPT group (124.4 ± 38.6) when compared to that of the control group (62.2 ± 17.4) (p=0.001, *Fig. 1*). P-selectin was found on a few endothelial cells but was higher in HPT group (110.4 ± 34.4) than in control group (36.0 ± 12.6) (p=0.001, *Fig. 2*). Although CT was widely distributed on HEV, the HPT group (285.8 ± 61.6) showed a heavier labeling when compared to that of the control group (93.4 ± 21.5) (p=0.002, *Fig. 3*).

Electron microscopic analysis displayed the migration of lymphocytes from blood through HEV *(Fig. 4)*. Close membranous contact was noticed between lymphocytes and endothelial cell of HEVs. Adjacent endothelial cells were detached from each other and junctional complexes disappeared in the migration areas of inter-endothelial routes *(Fig. 4)*. Lymphocytes migrating transendothelially invaginated into the cytoplasm of endothelial cells on HEVs.

DISCUSSION

HEVs are specialized post-capillary venules found in lymphoid organs and chronically inflamed tissues and are the sites for migration of lymphocytes from the blood vascular system to lymph nodal parenchyma. Endothelial cell surface components may be involved in the apparently selective interaction between lymphocytes and endothelial cells of HEVs (17). Different adhesion molecules have been reported to take part in the migration process, but their control mechanisms are not completely known. In this study, CT was shown to increase in the surface endothelium of HEVs of HPT group, and it was speculated that this molecule may regulate the migration process via increasing E-selectin and P-selectin of surface endothelial cells of HEVs.

Leukocyte migration from blood to tissues is a multi-step process and the route of migration of lymphocytes is through HEV (17). Our electron microscopic finding

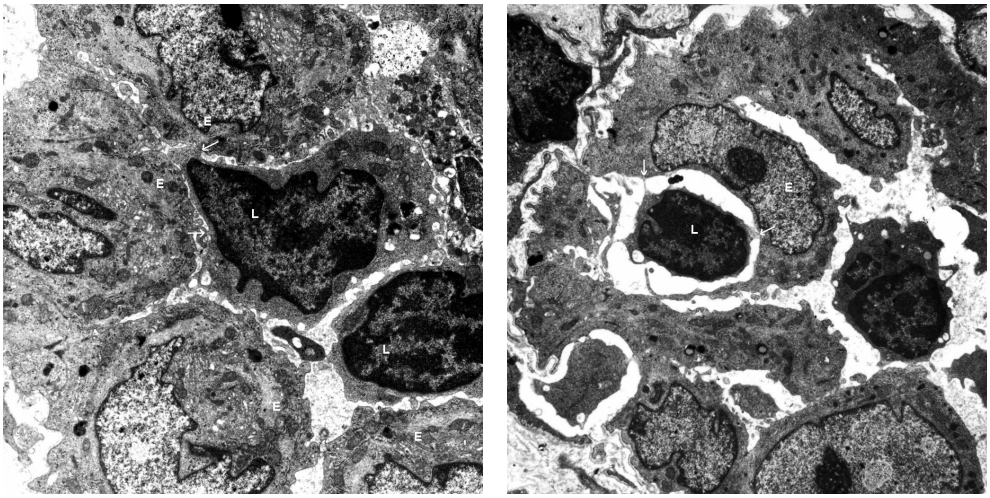

Fig. 4: (left) In the hypertrophic group, migration of lymphocytes (L) was seen between two endothelial cells (E) through the HEV. (right) Close cytoplasmic contact was noticed between lymphocyte and high endothelial cells (arrows). Original magnification X8800 (left) and X6000 (right).

demonstrated that lymphocytes migrate via the HEV although this matter has been disputed in the past (18). Additionally, we noticed that there are membranous contacts between lymphocytes and endothelial cells of HEV. Membranous contact in the form of cell-cell adhesion may be the morphological signal to initiate lymphocyte migration. It is known that lymphocytes that attached to HEVs remain loosely attached only a few seconds, detach, bind again and thus begin to roll on the endothelial surface. Meanwhile, chemokines are produced in the endothelial cells that encounter rolling lymphocytes, and this further increases their strength of attachment (6).

We investigated the immunohistochemical localization of E- and P-selectins of HEV in human pharyngeal tonsils and found that expression of these selectins was higher in HPT group compared to the control group. E-selectin and P-selectin are known to be expressed by endothelial cells (EC) in HPT (11), and the expression changes in response to proinflammatory stimuli was reported for human palatine tonsil by Andoh et al (19). Endothelial cell surface components are involved in the apparently selective interaction between lymphocytes and endothelial cells of HEVs (17). When T lymphocytes differentiate from effector cells in peripheral lymphoid organs, several adhesion molecules change. The expression of L-selectin decreases, and the levels of several integrins, ligands for E- and P- selectins and CD44, increase (6). The expression of E- and P-selectins that are produced in the endothelium of the HEV during infection help bind ligands expressed by T cells. It is known that several cell adhesion molecules mediate the binding of lymphocytes to HEV. Selectins are a family of adhesion molecules that mediate the initial adhesive step, and selectin-mediated cell-cell interaction is a prerequisite for subsequent firm attachment and transmigration of leukocytes (10,20,21).

Significant amounts of CT in the endothelium of HEV of HPT were documented for the first time by immunohistochemistry in this study and we speculated that increased CT raises intracellular calcium and controls expression of the Ca-dependent adhesion molecules, such as selectin, on surface epithelial cells thereby modulating lymphocyte migration. However, Huang reported similar mechanism for polymorpho-

nuclear leukocyte (PMN) that the transient increase in free calcium concentration of endothelial cell cytosol ($[Ca^{2+}]^i$) induced by PMN adherence to these cells was required for PMN transit across the endothelial cell barrier (22). Lorenzon et al determined that anti-P- selectin mAb and anti-E-selectin mAb, induced transient increases in EC $[Ca^{2+}]$ (23). Previous studies have shown that CT leads to a rise in intracellular calcium and/or cAMP, which in turn regulates cellular functions (24-26). Depending upon the target cell, the CTRs are known to couple to multiple heterotrimeric G proteins, leading to the activation of adenyl cyclase and/or phospholipase C (24-26). Signaling through both cAMP and intracellular calcium are important in CT action in osteoclasts, chondrocytes, and renal cells. In brain, however, CT apparently causes no change of adenylate cyclase activity, and there is some evidence that calcium acts as a second messenger in this tissue (27).

CT-induced increases in the level of E-selectin and P-selectin expression would increase endothelial adhesiveness between the lymphocytes and endothelial cells, and this scenario may have functional consequences for the process of migration of lymphocytes. The lymphocytes also need to adhere to the epithelial cells of the HEV and the up-regulation of E-selectin and P-selectin at the time of migration may lead to this adherence. The cell-cell contact, which may be the initial signal that ultimately leads to the migration of lymphocytes, could in part be the result of the CT-mediated up-regulation of E-selectin and P-selectin in endothelial cells. Therefore, we envision a role for CT in coordinating the migration of lymphocytes. In response to this hormone, the lymphocytes are ready to adhere as the endothelium becomes receptive and the junctions begin to open up. Future development of an in vitro model system to study the interactions between lymphocytes and endothelial cells of HEV would allow us to test this hypothesis more rigorously.

REFERENCES

1. Zidan, M, P Jeker, R Pabst: Differences in lymphocyte subsets in the wall of high endothelial venules and the lymphatics of human palatine tonsils. Scand. J. Immunol. 51 (2000), 372-376.
2. Steeber, DA, ML Tang, XQ Zhang, at al: Efficient lymphocyte migration across high endothelial venules of mouse Peyer's patches requires overlapping expression of L-selectin and ß7 integrin. J. Immunol. 15 (1998), 6638-6647.
3. Pabst, R, RM Binns: Heterogeneity of lymphocyte homing physiology: Several mechanisms operate in the control of migration to lymphoid and non-lymphoid organs in vivo. Immunol. Rev. 108 (1989), 83-109.
4. Picker, LJ, EC Butcher: Physiological and molecular mechanisms of lymphocyte homing. Ann. Rev. Immunol. 10 (1992), 561-591.
5. Miyasaka, M, T Tanaka: Lymphocyte trafficking across high endothelial venules: Dogmas and enigmas. Nat. Rev. Immunol. 4 (2004), 360-370.
6. Abbas, AK, AH Lichtman: *Cellular and Molecular Immunology*. Fiftieth Edition, Saunders, 2003, chapter 2, pp 34-39.
7. Tedder, TF, DA Steeber A Chen, et al: The selectins: Vascular adhesion molecules. FASEB J. 9 (1995), 866-873.
8. Gallatin, WM, IL Weissman, EC Butcher: A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature 304 (1983), 30-34.
9. Arbones, ML, DC Ord, K Ley, et al: Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin (CD62L) deficient mice. Immunity 1 (1994), 247-260.
10. Hirata, T, BC Furie, BP Furie, et al: L-selectin mediate migration of activated CD8+ T lymphocytes into inflamed skin. J. Immunol. 169 (2002), 4307-4313.
11. Perry, ME, WN Kirkpatrick, LC Happerfield, et al: Expression of adhesion molecules on the microvasculature of the pharyngeal tonsil (adenoid). Acta Otolaryngol. Suppl. 523 (1996), 47-51.
12. Fischer, JA, PH Tobler, M Kaufmann, et al: CT: Regional distribution of the hormone and its binding sites in the human brain and pituitary. Proc. Natl. Acad. Sci. 78 (1981), 7801-7805.
13. Ding, YQ, LJ Zhu, MK Bagchi, et al. Progesterone stimulates calcitonin gene

expression in the uterus during implantation. Endocrinology 135 (1994), 2265–2274.
14. Cavagna, M, JC Mantese: Biomarkers of endometrial receptivity - A review. Placenta 24 Suppl B (2003), S39-S47.
15. Foster, GV, A Baghdiantz, A Kumar, et al: Thyroid origin of calcitonin. Nature 202 (1964), 1303-1305.
16. Zhu, LJ, MK Bagchi, IC Bagchi: Attenuation of calcitonin gene expression in pregnant rat uterus leads to a block in embryonic implantation. Endocrinology 139 (1998), 330-339.
17. Indrasingh, I, G Chandi, S Vettivel: Route of lymphocyte migration through the high endothelial venule (HEV) in human palatine tonsil. Ann. Anat. 184 (2002), 77-84.
18. Schoefl, GI: The migration of lymphocytes across the endothelium in lymphoid tissue. A reexamination. J. Exp. Med. 136 (1972), 268-588.
19. Andoh, N, H Ohtani, C Kusakari, et al: Expression of E- and P-selectins by vascular endothelial cells in human tonsils. Acta Otolaryngol. Suppl. 523 (1996), 523-524.
20. Springer, TA. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. Ann. Rev. Physiol. 57 (1995), 827-872.
21. Vestweber, D, JE Blanks: Mechanisms that regulate the function of the selectins and their ligands. Physiol. Rev. 79 (1999), 181-213.
22. Huang, AJ, JE Manning, TM Bandak, et al: Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. J. Cell Biol. 120 (1993), 1371-1380.
23. Lorenzon, P, E Vecile, E Nardon, et al: Endothelial cell E- and P-selectin and vascular cell adhesion molecule-1 function as signaling receptors. J. Cell Biol. 7 (1998), 1381-1391.
24. Li, Q, J Wang, DR Armant, et al: Calcitonin down-regulates E-cadherin expression in rodent uterine epithelium during implantation. J. Biol. Chem. 277 (2002), 46447-46455.
25. Chabre, O, BR Conklin, HY Lin, et al: A recombinant calcitonin receptor independently stimulates 3',5'-cyclic adenosine monophosphate and Ca2+/inositol phosphate signaling pathways. Mol. Endocrinol.6 (1992), 551-556.
26. Force, T, JV Bonventre, MR Flannery, et al: A cloned porcine renal calcitonin receptor couples to adenylyl cyclase and phospholipase C. Am. J. Physiol. 262 (1992), F1110-F1115.
27. Twery, MJ, PK Seitz, GA Nickols, et al: Analogue separates biological effects of salmon calcitonin on brain and renal cortical membranes. Eur. J. Pharmacol. 155 (1988), 285-292.

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