Presence and possible role of vascular endothelial growth factor in thyroid cell growth and function

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Abstract

Angiogenesis is an important component in the development of thyroid goitre. Vascular endothelial growth factor (VEGF) represents a family of specific endothelial cell mitogens involved in normal angiogenesis and in tumour development. The purpose of this study was to determine the distribution of VEGF in thyroid tissues during goitre formation, and to study the actions of VEGF on the regulation of thymidine incorporation and iodine uptake by thyroid follicular cells. Goitre was induced in adult rats by administration of methimazole together with a low iodine diet. Thyroid from normal or goitrous rats was removed, fixed and sectioned. Immunocytochemistry performed for VEGF using the avidin–biotin system showed that VEGF is present in normal thyroid and is located mainly in the vascular endothelium and interfollicular stromal tissue. After administration of goitrogen for 2 weeks, which caused a two- to threefold increase in thyroid weight, staining of VEGF was less apparent within the interfollicular stroma, but strongly increased throughout the thyroid follicular and endothelial cells. Uptake of \(^{125}\)I and incorporation of \(^3\)H thymidine by Fisher rat thyroid cells (FRTL-5) were measured after 72 h culture with or without TSH or VEGF, or both. In the absence of TSH, incubation with VEGF caused a significant reduction in \(^3\)H thymidine incorporation, but did not significantly alter \(^{125}\)I uptake. Incubation with TSH (1 mU/ml) caused a fourfold increase in \(^3\)H thymidine incorporation that was diminished by co-incubation with 10 ng/ml or greater VEGF. Similarly, 10 ng/ml or greater VEGF significantly reduced the ability of TSH to increase \(^{125}\)I uptake. The antagonistic effects of VEGF on TSH-stimulated \(^3\)H thymidine incorporation or \(^{125}\)I uptake were significantly reduced in the presence of an anti-VEGF antiserum. A DNA fragment representing mRNA encoding the VEGF receptor, flt-1, was identified in FRTL-5 cells by reverse transcription PCR analysis, and the abundance of this fragment was increased in FRTL-5 cells cultured in the medium containing TSH (1 mU/ml) or fibroblast growth factor (FGF)-2 (25 ng/ml). These results indicated that VEGF and one of its receptors, Flt-1, are present in epithelial cells of the thyroid, and that VEGF could contribute to the regulation of development and function of thyroid epithelial cells.

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Introduction

Thyroid gland is well vascularized, with one of the highest blood flow rates per unit weight of tissue in the body. In pathological conditions such as Graves’ disease, thyroid enlargement and hyperfunction are accompanied by a markedly increased blood flow. Experimental models of goitre have shown that endothelial cells proliferate before follicular cells, perhaps because vascularization may be an important prerequisite for sustaining thyroid growth (Denef et al. 1989). During thyroid involution induced by iodide, there is a rapid contraction of capillary vascularization, again emphasizing the rapidity of response of the thyroid vasculature (Mahmoud et al. 1986, Wollman et al. 1990). Mechanisms mediating thyroid microvascular modification are not well understood. A number of growth and vasoactive factors are produced in thyroid and are considered to be potentially responsible for changes in thyroid microvasculature and blood flow (Dumont et al. 1992). We previously found that fibroblast growth factor (FGF)-2, a potent angiogenic protein, is synthesized and secreted by thyroid cells and, in cooperation with thyroid-stimulating hormone (TSH), regulates the growth and function of thyroid cells (Logan et al. 1992, Becks et al. 1994, Hill et al. 1994).

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein of 46 kDa which is a potent mitogen for endothelial cells both \textit{in vitro} and \textit{in vivo}, and also has vascular permeability-enhancing activity (Ferrara et al. 1992). VEGF is expressed in many tumours,
including human thyroid tumour cells (Inagaki et al. 1995), but is also expressed in normal keratinocytes, activated macrophages, renal mesangial cells and smooth muscle cells, suggesting a role in developmental and homeostatic, in addition to neoplastic, angiogenesis (Ferrara et al. 1992). This view is supported by the presence of VEGF mRNA and VEGF bioactivity in rat and human granulosa and theca cells, suggesting a contribution to the angiogenesis associated with the ovarian cycle (Koos 1986, Yan et al. 1993, Kamat et al. 1995). However, high levels of expression of VEGF are also detected around microvessels in areas where endothelial cells are normally quiescent (Ferrara et al. 1992). VEGF mRNA was identified in human thyroid follicles isolated from patients with Graves’ disease, and shown to be increased after exposure to TSH, Graves’ IgG, or insulin (Sato et al. 1995). VEGF mRNA was also detected within the thyroid gland of the rat 3 days after treatment with thiouracil to induce goitre. It therefore seems likely that VEGF is upregulated during goitre, possibly under the transcriptional control of TSH.

Two tyrosine kinase-type receptors, Flt-1 and Flk-1, have been shown to bind VEGF with high affinity (Fong et al. 1995, Sato et al. 1995). The organ distribution of flt-1 mRNA correlates well with the distribution of VEGF binding in adult tissues (Shibuya et al. 1990). Both flt-1 and flk-1 mRNAs were detected in rat thyroid after thiouracil induction of goitre (Sato et al. 1995). However, it is not clear whether VEGF and its receptors are expressed within endothelial cells or thyrocytes during the goitrous state, or whether both thyrocytes and endothelial cells are biologically responsive. The purpose of this study was therefore to examine the cellular distribution of VEGF during the formation of thyroid goitre in the rat, and to examine the possible biological actions of VEGF on rat thyrocytes.

Materials and Methods

Materials

Fisher rat thyroid (FRTL-5) cells (F2 sub-clone) were kindly provided by Dr Leonard D Kohn (Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD, USA). Materials were purchased from the following sources: transferrin, bovine insulin, bovine TSH, hydrocortisone, glycyl-histidyl-lysine, somatostatin, Coon’s modified F-12 M medium powder, non-essential amino acids, and ExtrAvidin peroxidase staining kit from Sigma Chemical Co., St Louis, MO, USA; 125I-labelled sodium iodide (IMS30) from Amersham International, Mississauga, Ontario, Canada; calf serum, trypsin, collagenase, Superscript II, RNase H reverse transcriptase, 123 bp DNA ladder, and Taq DNA polymerase from Gibco BRL, Burlington, Ontario, Canada. Recombinant human VEGF and anti-human VEGF neutralizing antibody were purchased from R&D System Inc., Minneapolis, MN, USA. RNase H reverse transcriptase, 123bp DNA ladder, and 2'-deoxynucleoside 5'-triphosphate were each purchased from Pharmacia Biotech Inc., Baie D’Urfé, QC, Canada.

Animals

All procedures had prior approval of the animal care committee of the University of Western Ontario and were performed in accordance with the guidelines of the Canadian Council for Animal Care. Young adult male Wistar rats weighting 250–275 g were obtained from Charles River Limited, StConstant, QC, Canada and housed with a 12 h light : darkness cycle. Forty rats were separated into two equal groups: one group received a low iodine formulation of rat chow (0·05 p.p.m.) that was isocaloric with normal diet, together with 0·01 (w/v) methimazole in their drinking water; the remaining animals formed a control group that received normal rat chow and tap water. Food and water were available ad libitum to both groups. Ten rats from each group were killed by CO2 asphyxiation after 1 week and the remainder were killed after 2 weeks. After rats were weighed, blood was collected by cardiac puncture and serum was prepared and frozen at −20°C until radioimmunoassay for thyroxine (T4) and tri-iodothyronine (T3). Thyroid glands were isolated, weighed, and fixed for later immunocytochemistry by immersion in 4% (w/v) paraformaldehyde–0·2% glutaraldehyde in 100 mM PBS for 24 h, followed by two washes in PBS, each for 24 h. Tissues were then placed in 30% (w/v) sucrose in PBS for 5 days before dehydration in 70% (v/v) ethanol and embedding in paraffin. All treatments were at 40°C.

Uptake of 125I] and incorporation of 3H]thymidine

FRTL-5 cells were cultured and passaged as described by Ambesi-Impimambato & Perrild (1989). Cells from passages 8–15 were used in these studies. Cells were cultured in 6H medium (F-12 medium containing TSH (0·1 IU/ml), hydrocortisone (0·4 ng/ml), transferrin (5 µg/ml), somatostatin (10 ng/ml), insulin (10 µg/ml), and glycyl-histidyl-lysine (10 ng/ml)) with 5% calf serum. The medium was changed every 3 days. Subconfluent cells were continually cultured in 5H medium (6H without TSH) containing 5% calf serum for 24 h, in 4H medium (5H without insulin) containing 5% calf serum for 24 h, and in 3H medium (transferrin, somatostatin and glycyl-histidyl-lysine) containing 2·5% calf serum for 24 h. Then cells were incubated in 3H medium without calf serum for 72 h. TSH (1 mIU/ml), VEGF or antibody against VEGF, were added alone or in combination, depending on the

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requirement of individual experiments. To estimate DNA synthesis, \[{}^3\text{H}\]thymidine (1 µCi/well) was added to cells for the final 24 h of culture. Cell layers were washed and solubilized by 0·1 M sodium hydroxide. The total DNA content of cells within individual wells was measured by fluorimetry, using Hoechst fluorochrome 33258, as described previously (Hill & DeSousa 1990). In separate experiments, cells were washed with PBS and then incubated with F-12 medium containing 100 000 c.p.m. carrier-free \(^{125}\)I-labelled sodium iodide for 30 min. \([{}^3\text{H}\] incorporation and \([{}^{125}\text{I}\)] uptake by cells were calculated as c.p.m./µg DNA.

**Immunocytochemistry**

Tissue sections (5 µm) in paraffin were prepared for immunocytochemistry and after rehydration in a descending ethanol series, and sections were incubated in 1% (v/v) hydrogen peroxide to block endogenous peroxide activity, followed by a 15 min incubation in 5% BSA in PBS to reduce non-specific binding. Slides were incubated for 48 h at 40 °C in a humidified chamber with a primary antibody against VEGF at a dilution of 1:1000 in PBS (pH 7·5) containing 2% (w/v) BSA and 0·01% sodium azide (100 µl per slide). All subsequent incubations were at room temperature. An appropriate biotinylated anti-IgG antibody diluted 1:500 in the same buffer was applied for 2 h, then slides were washed in PBS and incubated with avidin and biotinylated horseradish peroxidase for 1 h. After washing in PBS followed by 50 mM Tris–HCl (pH 7·5), the peptide immunoreactivity was localized by incubation in fresh 1·89 mM dianinobenzidine with 0·03% (v/v) hydrogen peroxide for 2 min and the reaction was quenched in excess 50 mM Tris–HCl (pH 7·5). Tissue sections were counterstained with Carazzi’s haematoxylin, dehydrated in an ascending ethanol series, cleared with xylene and mounted under glass coverslips. Negative controls included preabsorption of the primary antibody with VEGF and incubation of sections without primary or second antibody.

**Reverse transcription-PCR analysis**

To examine whether VEGF receptors were present in thyroid cells, FRTL-5 cells were cultured in 6H medium containing 5% calf serum to subconfluence. Subconfluent cells were furthered cultured for 3 days in serum-free 3H medium containing TSH (1 mU/ml) or FGF-2 (25 ng/ml) or in 6H medium containing 5% calf serum. After washing with ice-cold PBS buffer, total RNA was isolated from cells by acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski & Sacchi 1987). Total RNA was isolated from adult rat or mouse gut and intestine by RNAse Midi Kits following the instruction provided by the manufacturer. RT-PCR analysis was performed as previously described (Fong et al. 1995). In brief, the total RNA (10 µg) isolated either from FRTL-5 cells or from rat or mouse tissues was used as the template in each reverse transcription reaction, using dN6 random hexamer as primers. The conditions for reverse transcription were based on the protocol provided by Gibco-BRL. The amount of available template was further standardized by PCR amplification of the β-actin signal from each sample (not shown). The following oligonucleotides were used for the amplification of rat flk-1 and flt-1 using Taq DNA polymerase: sense rflt-1 (5’CATGGTCAGCTGC TGGGACACCGGC3’), antisense rflt-1 (5’GACTCCCT GCATCCTAACAATAT3’); sense rflk-1 (5’TCACTT GATCGGGGCAAGAGG3’), antisense rflk-1 (5’GGATCACCACAGT TGTCTTGTCTGTT3’). Separate primers were used for the amplification of flt-1 depicted in Fig. 6: sense (5’CGCGGTCTTGGCTTAC GCCT3’), antisense (5’CCATTATGGGGCTGCT TCCCCCGTCA3’). Pilot experiments were performed for the amplification of flk-1 and flt-1 sequences from the RNA extracted from rat tissues and the FRTL-5 cell line. These amplifications demonstrated linearity up to 35 cycles under our conditions. For the amplification of both flk-1 and flt-1, the following cycle parameters were used: 94 °C for 45 s, 62 °C for 30 s, and 72 °C for 2 min. The reactions were allowed to repeat for 35 cycles for flt-1 and 36 cycles for flk-1.

**Statistical analysis**

The differences in \([{}^3\text{H}\]thymidine incorporation and uptake of \([{}^{125}\text{I}\)] I by FRTL-5 cells between control and experimental groups were analysed by Student’s t-test. Each experiment was repeated at least three times. Representative photomicrographs of VEGF immunohistochemical distribution in rat thyroid are shown.

**Results**

After 2 weeks on a low iodine diet and receiving methimazole, rats demonstrated more than a doubling in thyroid weight, relative to body weight, compared with
VEGF could modulate the stimulatory effects of TSH-stimulated DNA synthesis and uptake of \([^{125}I]\) in cultured FRTL-5 cells. The effects of VEGF on TSH-stimulated DNA synthesis were inhibited in the presence of increasing concentrations of an antibody against VEGF, indicating that the presence of increasing concentrations of an antibody against VEGF was omitted in the incubation. Horizontal bar represents 75 μm.

**Discussion**

The coordination of thyroid epithelial cell growth with that of mesenchymal tissues such as endothelial and stromal cells is required for the homeostasis of thyroid gland in either physiological or pathological conditions. Whereas TSH is a major stimulus to thyrocyte iodide metabolism, growth of the thyroid involves interactions between TSH and locally derived autocrine or paracrine factors (Dumont & Vassart 1995). Multiple growth factors including FGF-2, transforming growth factor \(\beta\) (TGF\(\beta\)), epidermal growth factor (EGF) and the insulin-like growth factors (IGFs) and their associated binding proteins (IGFBPs) have been found in thyroid tissues. These growth factors have been implicated in the regulation of thyroid growth during goitre formation, and also modulate the metabolic effects of TSH on thyroid cells (Westerman et al. 1983, Bachrach et al. 1985, Morris et al. 1988, Wang et al. 1990, Cowin et al. 1992, Logan et al. 1992, Becks et al. 1994, Hill et al. 1994). Proliferation of endothelial cells, expansion of vascular spaces and increase in local blood flow are all hallmarks of iodine-deficient goitre (Denef et al. 1989). VEGF mRNA and that of its receptors were found to be present in the human and rat thyroid, and to be upregulated in the rat shortly after the induction of goitre (Sato et al. 1995). In this study, we have found that the distribution of VEGF is limited largely to the vascular endothelium in the normal rat thyroid, but is substantially altered in goitrous tissue, to include most thyrocytes. This supports a role for locally derived VEGF in the angiogenesis of goitre, but raises the question of whether endothelial cells alone are the target population, or if thyrocytes are also responsive.

The presence of both flt-1 and flk-1 mRNA has been reported in rat thyroid after the induction of goitre, although no mRNA was found in human thyroid from patients with Graves’ thyroïditis (Sato et al. 1995). The cellular location of these receptors was not identified, but a most obvious possibility would be their upregulation in thyroid endothelial cells. With a combination of immunohistochemistry for VEGF and RT-PCR to identify flt-1 and flk-1, we have obtained evidence that at least one of these receptors, Flt-1, is also expressed in thyroid epithelial cells. This surprising result suggests possible biological actions of VEGF on the thyrocyte population. Consistent with this idea, we found that VEGF, previously

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**Figure 1** Immunocytochemistry staining for VEGF in normal (A) and hyperplastic (C) rat thyroid sections, and controls (B and D). Arrows denote staining of the vascular endothelial cells in A, and of the hyperplastic thyrocytes in C. In the control sections the primary antibody against VEGF was omitted in the incubation. Horizontal bar represents 75 μm.
recognized as an endothelium-specific mitogen, inhibited both TSH-dependent DNA synthesis and iodide uptake by FRTL-5 cells. The mitogenic actions of VEGF on endothelial cells are mediated primarily by interactions with Flk-1 (Waltenberger et al. 1994). The probable mediation of the growth-inhibitory effects of VEGF on thyrocytes in this study via the Flt-1 receptor suggests that the two VEGF receptors can induce opposite biological responses to the same ligand. The localization of VEGF immunoreactivity to endothelial cells in the normal rat thyroid may represent the association of ligand expressed by surrounding stromal cells with Flt-1 receptors, as little VEGF has been found to be expressed by endothelial cells in other tissues.

Sustained increases in serum TSH after administration of a goitrogen cause three phases of thyroid growth in rat: an initial 1–2 month period of rapid proliferation of follicular and stromal cells is followed by a long plateau phase during which little or no growth occurs, and finally, after 6–12 months, follicular cell tumours begin to appear.

Figure 2 Effect of VEGF on [3H]thymidine incorporation (expressed as c.p.m./μg DNA) by rat thyroid (FRTL-5) cells. Subconfluent FRTL-5 cells were cultured in serum-free 3H medium for 72 h. VEGF alone (a) or in combination with TSH (1 mU/ml) (b) was added to cultures in increasing concentrations. [3H]Thymidine (1 μCi/well) was added at the last 24 h incubation. *P<0.05 or better compared with control (a) or TSH alone (b); n=3.

Figure 3 Effect of VEGF on uptake of [125I] (expressed as c.p.m./μg DNA) by rat thyroid (FRTL-5) cells. Subconfluent FRTL-5 cells were cultured in serum-free 3H medium for 72 h. VEGF alone (a) or in combination with TSH (1 mU/ml) (b) was added to cultures in increasing concentrations. *P<0.05 or better compared with control (a) or TSH alone (b); n=3.
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(Wynford-Thomas et al. 1983). An increased presence of VEGF mRNA occurs in the rat thyroid within 3 days of the induction of goitre and has been directly linked to the presence of increased concentrations of TSH (Sato et al. 1995). This would coincide with a rapid proliferation of endothelial cells required to support goitre formation, but also with a hyperplasia of thyrocytes. It is possible that VEGF provides a short-loop feedback system to limit thyrocyte proliferation, and to maintain a balance between thyrocyte mass and the expanding capillary network. It may contribute to the subsequent plateau of thyroid growth seen after goitre is induced in vivo. In this role VEGF, would oppose the actions of locally produced FGF-2 and IGF-I, which are also increased in abundance during goitre in rats, but which partly mediate the mitogenic actions of TSH (Becks et al. 1994, Hill et al. 1994, Phillips et al. 1994). An inhibitory influence of VEGF on thyrocyte proliferation would be additive to the effects of locally produced TGFβ (Logan et al. 1994). Interactions may exist between the expression of VEGF and the presence of other growth factors during goitre, as VEGF expression was induced in fibroblast and epithelial cells in response to TGFβ (Pertovaara et al. 1994). The relationship between FGF-2 and VEGF is unknown, but demonstration of the presence of flt-1 mRNA in FRTL-5 cells by RT-PCR in this study suggests that the amplification signal for flt-1 is greater after the exposure of cells to either FGF-2 or TSH.

The intracellular second-messenger signals that mediate the mitogenic actions of VEGF on endothelial cells are unique. While autophosphorylation of Flt-1 on endothelial cells induced the phosphorylation of phospholipase-C gamma and GTPase-activating protein complex on both endothelial cells and transfected NIH-3T3 cells, only in the former did this result in cell proliferation (Seetharam et al. 1995). The second-messenger signals that might mediate the ability of VEGF to inhibit TSH-dependent DNA synthesis or uptake of iodine by FRTL-5 cells by RT-PCR in this study suggests that the amplification signal for flt-1 is greater after the exposure of cells to either FGF-2 or TSH.

In summary, VEGF and its specific receptor, Flt-1, are found in thyroid cells, and the presence and distribution of VEGF in thyroid tissues is increased during goitre formation. VEGF reversed the stimulatory effect of TSH on iodine uptake and DNA synthesis in FRTL-5 rat thyrocytes. These observations indicate that VEGF could be involved in the local regulation of thyrocyte growth.
proliferation and differentiation. The interactions of TSH with VEGF through different receptors and their associated second-messenger systems during goitre formation remain to be investigated.

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