Early Coronary Angiogenesis in Response to Thyroxine Growth Characteristics and Upregulation of Basic Fibroblast Growth Factor

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Abstract—Although a substantial coronary angiogenesis occurs after thyroid hormone treatment, its regulation and relationship to cardiac hypertrophy are not understood. This study was designed to determine (1) the onset of capillary proliferation, (2) the sites of capillary proliferation, and (3) whether basic fibroblast growth factor (bFGF) upregulation occurs in response to thyroxine administration. Male Sprague-Dawley rats were injected daily with l-thyroxine (T_4, 0.2 mg/kg SC). Bromodeoxyuridine labeling of capillary endothelial cells increased during the first 24 hours of treatment and peaked after 2 days of treatment. Northern blot analysis revealed a slight increase in bFGF mRNA during this period, followed by a doubling of expression by 48 hours, at which time bFGF protein was also increased. In situ hybridization, used to localize bFGF mRNA, showed an increase in transcripts within 24 hours after T_4. This enhancement was uniform in the epicardium and endomycocardium. Histochemical analysis (double staining for alkaline phosphatase and dipeptidyl peptidase) of frozen sections, used to discriminate capillary profiles as arteriolar and venular, respectively, showed that growth occurred in the latter, since the percentage of capillary profiles positive for dipeptidyl peptidase was higher than the control value after 4 days of T_4 administration. These data indicate that in the thyroxine model of cardiac hypertrophy (1) capillary DNA synthesis occurs after a single injection of thyroxine, (2) capillary growth coincides with an upregulation in bFGF mRNA and increase in bFGF protein, and (3) proliferation occurs in the venular capillaries. (Circ Res. 1998;82:587-593.)

Key Words: cardiac hypertrophy in situ hybridization basic fibroblast growth factor mRNA capillary microcirculation

Cardiac hypertrophy in response to chronically elevated thyroid hormone levels constitutes a model of volume overload leading to eccentric hypertrophy.1–10 In contrast to pressure-overload hypertrophy, which is often associated with decrements in ventricular function,1 hearts enlarged by thyroid hormone administration have enhanced left ventricular systolic function, including elevated maximal stroke volume1,2 and peak cardiac index.3 Thus, the hearts of hyperthyroid rats exhibit compensated ventricular function, a finding that may be related in part to substantial growth of coronary microvessels that has been documented by many studies.2,5–10 We have previously shown that maximal myocardial perfusion is normal or increased in this model.5,9 It is not surprising that this marked angiogenic response is not typical of other models of ventricular enlargement, since the stimulus evoking the cardiac hypertrophy is an important determinant of the response of the coronary vasculature.11 Administration of thyroid hormones causes a number of acute changes in cardiac function, including tachycardia, and increases in cardiac output, rate of change in left ventricular pressure (dP/dt), developed tension, and velocity of shortening (reviewed in References 12 and 13). As a consequence of the increased metabolism, oxygen consumption and coronary flow are markedly elevated.14 Enhanced blood flow or increased metabolites linked to increased flow may provide the initial signal for angiogenesis.15 We previously demonstrated that intermittent increases in coronary flow after dipyridamole injection are associated with a 33% increase in coronary capillary length density in rabbits developing cardiac hypertrophy due to hypertension.15

Recent evidence from our laboratory suggests that coronary angiogenesis is not a response to cardiac hypertrophy evoked by thyroxine but that it occurs before significant ventricular enlargement.2 The present study explored the initiation of angiogenesis in this model by testing three hypotheses. Our first hypothesis was that capillary endothelial cell DNA synthesis is initiated during the first 2 days of thyroxine treatment. The second hypothesis was that bFGF mRNA expression is markedly enhanced in response to thyroxine treatment and thereby may serve as a mediator for angiogenesis. This hypothesis is based on our finding that bFGF mRNA expression is markedly enhanced during the early period of fetal coronary vascularization and during the early neonatal period when capillary proliferation is particularly high.17 At least three other findings provide a rationale for this hypothesis. First, exogenous bFGF has been
shown to increase the number of capillaries and arterioles on the surface of infarcted rat hearts and to enhance collateral flow in the ischemic canine heart. Second, migration of endothelial cells from bovine coronary arteries is enhanced by bFGF and inhibited by anti-bFGF antibodies. Third, cell migration from embryonic hearts onto a collagen gel is enhanced when bFGF is added to the culture medium.

The third hypothesis was that angiogenesis in the hyperthyroid model occurs preferentially in the venous capillary bed. Rationale for this hypothesis are based on (1) our finding that the relative proportion of venular capillaries is higher in rats 1 month after birth than at 2 months of postnatal life and (2) evidence that angiogenesis occurs in both capillaries and venules. Therefore, a possible explanation of the relatively higher proportion of venous capillaries during the neonatal period is that angiogenesis occurs preferentially in the venous capillary bed.

Materials and Methods

Protocol

T4 (0.2 mg/kg SC) or saline was administered to male Sprague-Dawley rats for a period of 1 to 4 days. The day after the last injection, the rats were anesthetized with ketamine (50 mg/kg) and hearts from treated and control rats that were fixed with 2% paraformaldehyde on the Langendorff-type apparatus described in protocol 1, processed, and embedded in ParaPlast. Six-micrometer cross sections, stained with Gill’s hematoxylin, from these hearts were used. To determine the extent of DNA synthesis in capillary endothelial cells, rats were injected twice (100 mg/kg in 0.1 mol/L IP PBS), 2 hours apart, with BrdU (Sigma Chemical Co), a thymidine analogue that is incorporated in the S phase of proliferating cells. Two hours after the second dose, the rat was anesthetized with ketamine, and the heart was rapidly excised and frozen in OCT compound in isopentane precooled to −80°C. Sections, 4 µm thick, were prepared with a cryostat, placed on polylysine-coated slides, and fixed in 70% ethanol in glycine buffer precooled to −80°C. The sections were then incubated with BrdU primary monoclonal antibody (BrdU detection kit 1, Boehringer-Mannheim). Subsequently, the tissue sections were incubated with the secondary antibody (fluorescein-labeled sheep anti-mouse Ig) at 37°C. To indicate all nuclei, the tissues were stained with bisbenzimide H33258 for 15 minutes at room temperature.

To distinguish the arteriolar and venular portions of the capillary bed, we used 13-µm-thick sections cut on a cryostat to localize alkaline phosphatase and DPP IV, respectively. General histological analysis was based on sections (2 µm in thickness) from JB4-embedded specimens, which were stained with hematoxylin and eosin. To determine more subtle cellular and extracellular alterations, thin sections of Spurr epoxy resin–embedded specimens were cut with a diamond knife on a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7000 transmission electron microscope.

In Situ Hybridization

This procedure is identical to that recently published by us. A 395-bp fragment of the coding sequence of the rat bFGF was cloned into the pGEM 3Zf vector (Promega). The sequence corresponded to nucleotides 603 to 998 of the published clone of Shimazaki et al. Sense and antisense RNA transcripts incorporating [35S]UTP were produced after restriction digestion using EcoRI and HindIII, respectively. The deparaffinized tissue sections were hybridized overnight as described below.

After proteinase K (1 mg/mL) treatment at 37°C for 30 minutes, sections were incubated overnight with 50 µL hybridization buffer (0.3 mol/L NaCl, 20 mmol/L NaAc, 1 mmol/L EDTA, 0.4 mol/L dithiothreitol, 1× Denhardt’s–0.02% Ficoll, 0.02% polyvinylpyrrolidone, 250 µg/mL yeast tRNA, 10% dextran sulfate, and 50% formamide) containing 3.6×10^6 cpm riboprobe at 50°C. Sections were then treated with 20 µg of RNase A per milliliter and 1 U RNase T1 per milliliter at 37°C for 15 minutes. After they were washed, the slides were dehydrated by immersion in graded ethanol containing 0.3 mol/L ammonium acetate. The sections were air-dried, coated with NTB-2 nuclear tract emulsion, and stored in the dark with desiccant at 4°C for 2 to 3 weeks. After development, the sections were counterstained with hematoxylin and coverslipped with mounting medium.

Quantification of DNA Labeling

In preliminary experiments, we determined that nearly all of the DNA synthesis in T4-treated rats occurs in endothelial cells. These data were obtained from perfusion-fixed hearts from rats that had been injected with either BrdU or [3H]thymidine before they were killed for study. Slides from these rats were viewed with an oil-immersion lens, and the fraction of labeled nuclei was determined. This method has the advantage of discriminating cell types. We found that nonmyocyte nuclei constituted 63% of the nuclei in an average field (43% are endothelial and 20% are interstitial cells consisting primarily of fibrocytes). In order to include large numbers of nuclei (~1200 per slide), we first counted nuclei labeled with BrdU using a ×40 objective and then photographed the same fields using an ultraviolet cube to detect nuclei with the bisbenzimide stain. To be certain that the nuclei labeled with BrdU were nonmyocytes, the field was viewed alternatively with polarized light, allowing us to see the myocyte profiles. In order to express the number of labeled nuclei per nonmyocyte nuclei, we multiplied the total number by 0.63. Thus, the number of labeled nuclei is expressed per total nonmuscle nuclei. Since we found that BrdU-positive nuclei are almost all from endothelial cells, the percent labeled endothelial cells is an underestimation.

Northern Blot Analysis

Total RNA was prepared from staged prenatal and postnatal rat heart tissue using the guanidinium thiocyanate method of Chomczynski and Sacchi. The samples were prepared on 1.2% agarose gels in MOPS buffer containing 6% formaldehyde. The separated RNAs were transferred overnight onto nylon membrane by capillary action using 25 mmol/L phosphate buffer, pH 6.3. Completeness of transfer was checked by viewing the gel under UV light. The RNA transferred onto the membrane was UV–cross-linked and then prehybridized at 42°C in 50% formamide, 5× SSC (750 mm sodium chloride and 75 mmol/L sodium citrate, pH 7), 5× Denhardt’s solution, 10% SDS, 5% dextran sulfate, and 20 µg/mL freshly denatured salmon sperm DNA. After 1 hour, 4×10^6 cpm/mL of freshly denatured [3P]labeled DNA prepared by antisense transcription, as described for in situ hybridization, was added to the hybridization solution. The membranes were hybridized overnight at 55°C and then washed twice in 2× SSC and 0.2% SDS and twice in 0.1× SSC and 1% SDS at 50°C. The dried membranes were exposed to Kodak X-OMAT film. After development, the quantity of mRNA was standardized to hybridization.

Selected Abbreviations and Acronyms

- bFGF = basic fibroblast growth factor
- BrdU = 5-bromo-2’-deoxyuridine
- DPP IV = dipeptidyl (amino) peptidase IV
- T4 = 1-thyroxine
- VEGF = vascular endothelial growth factor

- **DPP IV**: dipeptidyl peptidase IV
- **VEGF**: vascular endothelial growth factor
- **BrdU**: 5-bromouridine
- **Eco RI**: restriction enzyme
- **Hin dIII**: restriction enzyme
tion of the same film to 28S RNA, which served as an internal standard.

Western Blot Analysis

Western blotting was used to estimate bFGF protein in the left ventricular samples. Frozen tissue samples were homogenized in Tris-EDTA buffer containing protease inhibitors. The homogenate was centrifuged at 20,000g for 90 minutes, and the resulting supernatant was applied to a heparin-Sepharose affinity column. bFGF was eluted with 2 mol/L NaCl–10 mmol/L Tris, pH 7. This material was concentrated, and protein levels were determined and separated on a 15% SDS-polyacrylamide gel. The protein bands were transferred to nitrocellulose, blocked with 5% powdered milk, and incubated overnight with fibroblast growth factor-2 rabbit polyclonal antibody diluted 1:400 (Santa Cruz Biotechnology). The nitrocellulose was washed and blocked as described above and incubated for 1 hour with anti-rabbit IgG conjugated to horseradish peroxidase. After further washing, the nitrocellulose was incubated for 1 minute with the ECL detection reagent (Amersham Life Sciences) and exposed to Kodak X-OMAT x-ray film for 2 minutes to permit visualization of the protein bands.

Statistical Analysis

Data are presented as group means (±SEM) and were analyzed using a one-way ANOVA and a Bonferroni adjustment for multiple comparisons, with P≤.05 selected to denote statistical significance. Because of unequal variances for the DNA synthesis data, we used log transformation to reduce the heterogeneity of variance between groups. An ANOVA was performed on the transformed values, and the means presented are to the original scale.

Results

Early Onset of Capillary Proliferation

To determine whether histological or cytological changes occurred in response to early thyroxine treatment, we examined tissues from rats treated for 2 or 4 days. Degenerative changes were not observed with the light microscope. In cross-sectional fields of rats treated for 4 days, we did note the presence of capillary profiles within cardiac myocytes. With the electron microscope such capillary profiles could be seen as being surrounded by a single myocyte profile (Fig 1). Thus, the capillary growth associated with thyroxine treatment includes infolding of the cardiocyte cell membranes, allowing the adjacent capillary to lie closer to the cell center.

All data illustrated in Fig 2 are from rats treated with thyroxine for 4 days. The ratio of heart weight to body weight in these rats was elevated by 16%. Profiles that were from the arteriolar and venular ends of the capillary bed (based on alkaline phosphatase and DPP IV enzyme histochemistry) were quantified in tissue sections. The percentage of DPP IV (venular)–positive capillaries increased slightly but significantly in both the epimyocardium (7%) and endomyocardium (8%) after 4 days of thyroxine treatment. In both areas of the myocardium, there was no overlap between the treated and control individual values. Since capillary numerical density was unchanged, overall capillary growth kept pace with the myocardial hypertrophy, and this growth can be accounted for by proliferation of the venular portion of the capillary bed.

DNA synthesis of endothelial cells, determined by BrdU labeling, increased significantly during the first 24 hours after T4 administration, as noted by a nearly 5-fold increase over the control value (Fig 3). After 2 days, BrdU labeling peaked, with a 9-fold increase. By 4 days, the values were identical to those observed at 1 day. Thus, endothelial DNA synthesis is a relatively early response to thyroxine administration and supports the observed increase in capillarity noted at 4 and 5 days.

bFGF Expression During Early Thyroxine Treatment

Quantification of bFGF mRNA was determined by densitometric scanning of autoradiograms (Fig 4). During the first 24 hours after thyroxine administration, bFGF mRNA tended to increase (15%), and by 48 hours, it doubled. Thus, an increase in message for this growth factor occurs relatively early during
thyroxine administration, and the increase at 48 hours corresponds to the peak endothelial DNA synthesis shown in Fig 3.

In situ hybridization experiments revealed a fairly uniform labeling throughout the myocardium (Fig 5). To demonstrate uniformity of grain distribution throughout the left ventricle, we compared counts from the four fields used to obtain mean counts. Mean standard deviations were 21% and 16% in the control and treated groups, respectively. Endomyocardial and epicardial fields were similar with respect to labeling. Grain counts (counts/1000 \( \mu \)m\(^2\); mean\( \pm\)SEM) demonstrate an increase in transcripts with thyroxine treatment: rats treated for 1 day (n = 8), 32.8\( \pm\)3.3 counts/1000 \( \mu \)m\(^2\); control rats (n = 7), 20.8\( \pm\)3.9 counts/1000 \( \mu \)m\(^2\) (\( P < .03 \)). In two hearts from rats treated for 2 days, the values were 42.1 and 53.8 counts/1000 \( \mu \)m\(^2\). These in situ data are very consistent with the findings from Northern analysis.

bFGF Protein

Western blot analysis of left ventricles is provided in Fig 6. Three isoforms of \( \approx \)18, 21, and 22 kD were observed in all experiments and in samples from both control and T\(_4\)-treated rats. The density of the 18-kD isoform was less after 1 day of treatment but then rose to control levels after 2 days of treatment. After 4 days of treatment, protein levels for this isoform remained relatively constant or decreased slightly. Both the 21- and 22-kD isoforms were highest after 2 days of T\(_4\) administration. The density of these bands was 2- to 3-fold higher than that in the control rats. This increase did not persist, since the two bands after 4 days of T\(_4\) were similar to the control bands.

Discussion

A marked angiogenic response within the myocardium of rats chronically treated with thyroid hormones has been previously documented.\(^4\)\(^-\)\(^10\) However, the present study is the first to document the initiation and regulation of angiogenesis. These new findings support several conclusions. First, since DNA synthesis in capillary endothelial cells begins within 24 hours of thyroxine administration, capillary angiogenesis is an immediate response to some acute effect of elevated thyroxine levels. Our second conclusion is that bFGF may play a role in the angiogenic response, since both its mRNA and protein are increased during the first 48 hours after thyroxine administration.
tion. Finally, we have documented that proliferation occurs at the venular end of the capillary bed.

**Hemodynamic and Metabolic Factors as Primary Angiogenic Stimuli**

A substantial coronary capillary growth that matches the magnitude of left ventricular enlargement has been shown to occur within 5 to 10 days of thyroid hormone treatment in rats \(^1\) and pigs. \(^2\) In addition, other studies have shown that capillary growth parallels or exceeds left ventricular hypertrophy when thyroid hormones are administered for a longer period of time. \(^3,4\) Moreover, the angiogenic response includes resistance vessels, since arteriolar density and maximal coronary flow \(^5,6,7\) are normal. Our recent work documented a substantial capillary angiogenesis during the first 5 days of treatment, i.e., a 27% increase in length density in the epimyocardium. \(^8\) This finding suggested that the capillary growth was not a response to cardiac hypertrophy, since left ventricular mass was not, as yet, significantly elevated. The present study indicates that angiogenesis is initiated early and thus appears to be triggered by the metabolic effects of acutely elevated thyroxine levels.

Several lines of evidence indicate that the hypertrophic and angiogenic responses to thyroxine are secondary to its hemodynamic and metabolic effects. First, cardiac hypertrophy does not occur in heterotopically transplanted, unloaded hearts whose hosts were treated chronically with thyroxine. \(^9\) Similarly, we have demonstrated that neovascularization in fetal rat hearts, grafted in oculo, where they beat but exist in the unloaded state, is similar in euthyroid, hyperthyroid, and hypothyroid rats. \(^10\) Finally, we have shown that coronary capillary growth is not compromised by the hypertrophic state in rabbits. \(^11\)

Enhancement of coronary flow (or factors associated with such an increase) appears to provide a mechanical stimulus for capillary growth. This thesis is supported by work that documents capillary growth in response to chronic dipyridamole administration in rabbits \(^12\) and rats. \(^13\) Similarly, adenosine or HWA-285, a xanthine derivative, have been found to enhance coronary capillary growth. \(^14\) Although adenosine may also directly stimulate endothelial cell proliferation, as demonstrated by in vitro experiments, \(^15\) there is evidence that endothelial cells undergo DNA synthesis or proliferation when they flatten \(^16\) or are subjected to increases in flow or turbulence. \(^17\) Since increased diastolic filling characterizes the thyroxine model, the resulting stretch of the coronary vasculature may provide another mechanical stimulus for microvascular growth. This hypothesis is consistent with data showing that volume-overload-induced cardiac hypertrophy is associated with a proportional growth of capillaries and arterioles. \(^18\) Thus, the initial stimulus for a cascade of events that lead to neovascularization in this model is likely to be related to mechanical forces.

**Angiogenesis in the Venular Capillary Bed**

Using a dual histochemical procedure, \(^19,20,21\) we were able to document that growth occurs at the venular capillary bed. Alkaline phosphatase is concentrated in the arteriolar capillaries, whereas the proteinase DPP IV characterizes venular capillaries and small venules in many species, including rats and humans. \(^22\) Thus, in longitudinal sections of myocardium, one can visualize a transition of staining along the course of a capillary bed. \(^23,24\) The significant increase in DPP IV–positive capillary profiles noted in both the epimyocardium and endomyocardium of thyroxine-treated rats documents proliferation of the venular capillaries. This finding is consistent with evidence that DPP IV plays a role in angiogenesis, as evidenced by a decline in angiogenesis when this enzyme is inhibited. \(^25\) Capillary numerical density in both myocardial regions was nearly identical in rats treated with thyroxine for 4 days and their nontreated controls. There was a 16% increase in heart mass in the treated group; thus, a significant capillary growth occurred and can be accounted for by the increase in venular capillary profiles.

**Role of bFGF in Angiogenesis**

bFGF is a known mitogen for a variety of cell types of mesodermal and neuroectodermal lineages. \(^26\) Therefore, theoretically, a number of cell types, in addition to endothelial cells, could be targeted by this growth factor. However, cardiac myocytes are not known to proliferate in response to thyroid hormones, and fibroblast hyperplasia is unlikely, since fibrosis is not characteristic of this model. We have recently shown that bFGF transcripts are highest in the myocardium at two time points of development: (1) embryonic days 14 to 15 and (2) during the first week of postnatal life. \(^27\) These times correspond to an early period of vascularization and to a period of marked capillary proliferation, respectively. Additionally, in another study, we showed that migration distance and proliferation of undifferentiated cells and angioblasts from explanted embryonic hearts are enhanced with exogenous bFGF in a dose–dependent manner. \(^28\) Our present data show that upregulation of bFGF mRNA occurs during the first 48 hours of thyroxine treatment and that the high expression seen at 2 days corresponds to peak DNA synthesis in endothelial cells. Moreover, bFGF protein is also highest at this time.

Heretofore, the studies that reported coronary vascular growth in response to exogenous bFGF used models of ischemia. \(^29,30,31\) Moreover, pretreatment of isolated rat hearts with bFGF has a protective effect when the hearts are subjected to global ischemia. \(^32\) The presence of three bFGF peptide isoforms in heart tissue is consistent with an earlier study. \(^33\) That study reported an overall 1.5-fold increase in bFGF in hearts from rats that were administered thyroxine for 4 weeks. Although our data are consistent with this finding, they are based on the onset of thyroxine–stimulated growth, whereas the hearts included in the earlier study most likely were in a stage of limited growth. Thus, they found that the enhancement of bFGF protein was due mainly to an increase in the 18-kD peptide, whereas our increase at 2 days was attributable mainly to a 2- to 3-fold increase in the 21- and 22-kD peptides. The initial decrease of the 18-kD isoform that we observed may be due to its degradation. Mechanical activity may be the stimulus for release of the cytosolic 18-kD isoform of bFGF, since isolated paced ventricular myocytes have been shown to release more of this peptide into the culture medium. \(^34\) This increase in release was associated with greater sarcolemmal permeability. The myocytes in that study \(^35\) were
undergoing hypertrophy that could be blocked by the addition of neutralizing bFGF to the medium. Thus, bFGF may be playing a similar role in our thyroxine model. However, because this growth factor is a major mitogen for endothelial cells and its enhancement corresponds to endothelial cell proliferation, we conclude that it likely plays a role in early angiogenesis in this model.

Although our data show an upregulation of bFGF in response to thyroxine administration, this growth factor may not be singularly affected. Other growth factors cannot be excluded as potential regulators in this model. For example, previous work has shown that bFGF and VEGF have a synergistic effect on the proliferation of bovine capillary endothelial cells. Moreover, recent evidence has shown that the exon 6-encoded sequence of VEGF<sub>189</sub> exerts its effects through bFGF signaling pathways. Accordingly, we do not necessarily view bFGF as the sole mediator of angiogenesis. However, our data indicate that increases in this growth factor coincide with capillary endothelial DNA synthesis; therefore, this mitogen shows an important temporal relationship to the initiation of angiogenesis in response to thyroxine.

Conclusions

The present study explored capillary angiogenesis during the early period of thyroxine administration. On the basis of several approaches, we conclude that capillary proliferation is initiated during the first day of thyroxine treatment, occurs primarily in the venular capillaries, and may be regulated by bFGF.

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