Oral Diseases (2004) 10, 179–185

http://www.blackwellmunksgaard.com

Oral and Maxillofacial Pathology

The roles of vascular endothelial growth factor and angiopoietin-2 in the regression of pregnancy pyogenic granuloma

K Yuan[1,2], MT Lin[2]

[1]*Periodontics Division, Department of Dentistry, National Cheng Kung University Hospital;* [2]*Department of Biochemistry and Institute of Basic Medical Sciences, Medical School, National Cheng Kung University, Tainan, Taiwan*

OBJECTIVES: The molecular mechanism for the regression of pregnancy pyogenic granuloma after parturition remains unclear. It has been proposed that, in the absence of vascular endothelial growth factor (VEGF), angiopoietin-2 (Ang-2) causes blood vessels to regress. Therefore, we investigated the roles of Ang-2 and VEGF in the regression of pregnancy pyogenic granuloma.

MATERIALS AND METHODS: The effects of tumor necrosis factor-α (TNF-α) on the transcription of Ang-2 were tested in endothelial cells by reverse transcriptase-polymerase chain reaction. A total of 15 specimens, including granulomas taken from five gravidas during pregnancy, five after parturition, and five from normal gingiva were compared by immunoblot assays for their relative expressions of Ang-1, Ang-2, Tie-2, VEGF, and β-actin. Double staining, immunohistochemistry for Ang-2, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling for apoptotic cells, were used to evaluate their regression. Finally, a fibrin gel culture system was used to investigate whether the withdrawal of VEGF and addition of Ang-2 could cause newly grown microvessels to regress.

RESULTS: TNF-α upregulated the expression of Ang-2 in all endothelial cell types tested. The protein levels of Ang-2 and Tie-2 were highest in the granulomas in pregnancy, followed by those after parturition and normal gingiva, while Ang-1 and β-actin exhibited no significant differences. The amount of VEGF was high in the granulomas in pregnancy and almost undetectable after parturition. Double staining on granulomas after parturition revealed more apoptotic cells and less Ang-2 than did those in pregnancy. In the fibrin gel assay, VEGF alone or in combination with Ang-2 could protect microvessels from apoptosis, while Ang-2 alone had no effect.

CONCLUSIONS: Our findings suggest that a lack of VEGF is associated with apoptosis of endothelial cells and regression of granuloma. The roles of Ang-2 require additional study.
Oral Diseases (2004) **10**, 179–185

Keywords: pyogenic granuloma; angiogenesis; vascular endothelial growth factor; angiopoietin-2

Introduction

Partial or complete regression after parturition is one of the clinical features of pregnancy pyogenic granuloma (pyogenic granuloma in pregnancy; pregnancy epulis; lobular capillary hemangioma) (Seymour, 1992). However, the underlying molecular mechanism remains obscure. Periodontal surgery is relatively contraindicated during pregnancy (Little, 1988), so a better understanding of the molecular mechanism may help to develop non-surgical methods to control the disease. In a previous study, we found that angiogenic factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), were detected in significantly greater amounts in pyogenic granulomas in pregnancy than in those after parturition, in which VEGF and bFGF could be detected only in minimal amounts. On the contrary, tumor necrosis factor-alpha (TNF-α) levels in granulomas were higher after delivery (Yuan *et al*, 2002). This was consistent with other studies, which also indicated that TNF-α was upregulated after the withdrawal of female sex hormones (Pacifici *et al*, 1991; Tabibzadeh *et al*, 1999).

The downstream molecules of TNF-α signaling are very complex. One of them is angiopoietin-2 (Ang-2), which has been proposed to play a very important role in vascular morphogenesis (Hanahan, 1997; Kim *et al*, 2000a). Ang-2 and angiopoietin-1 (Ang-1) are two ligands for an endothelium-specific receptor tyrosine kinase, tyrosine kinase with Ig and EGF homology domains (Tie-2) (Maisonpierre *et al*, 1997). It was originally hypothesized that, in the absence of angiogenic factors (e.g. VEGF or bFGF), Ang-2, acted as the competitive antagonist of Ang-1 for binding to Tie-2

Correspondence: Ming T. Lin, Department of Biochemistry and Institute of Basic Medical Sciences, Medical School, National Cheng Kung University, no. 1 University Road, Tainan 701, Taiwan.
Tel: 886 6 235 3535 ext. 5544, Fax: 886 6 274 1694, E-mail: Lin1223@mail.ncku.edu.tw
Received 12 May 2003; revised 13 November 2003; accepted 15 December 2003

and caused blood vessel regression and the detachment of supporting cells from vessel walls (Hanahan, 1997). However, unequivocal reports have recently emerged to challenge the inhibitory role of Ang-2 in angiogenesis (Asahara *et al*, 1998; Kim *et al*, 2000b). Therefore, we were very interested to know the underlying mechanisms for the natural regression of pyogenic granulomas in pregnant women. Whether, and to what extent, can overproduction of Ang-2 (modulated by TNF-α) and a lack of VEGF contribute to the regression of the lesions? With more understanding of the molecular mechanisms, innovative and non-surgical therapeutic methods can be developed to reduce the surgical risk and discomfort on pregnant females.

Material and methods

Reverse transcriptase-polymerase chain reaction

In order to test whether TNF-α can influence the expression of Ang-2 in different endothelial cells, human umbilical vein endothelial cells (HUVEC), umbilical artery endothelial cells (HUAEC), iliac artery endothelial cells (HIAEC), and saphenous vein endothelial cells (HSVEC) were purchased from a biotechnology company (Cambrex, Walkersville, MD, USA) and maintained in Endothelium Growth Medium (EGM; Cambrex) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells between passages 4 and 6 were used for this study. One day before the addition of TNF-α, the medium was changed from EGM to M199 containing 1% fetal calf serum (FCS). After two phosphate-buffered saline (PBS) washes, the cells were incubated for 6 h in M199 containing 1% FCS and varying concentrations of TNF-α (0–50 ng ml^{-1}) (PeproTech EC Ltd., London, UK) for 6 h. With an RNA extraction kit (RNA Extraction Kit; Viogene, Taipei, Taiwan), we collected the total RNA, of which 100 ng was reverse-transcribed with an Reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Advantage^{TM} RT-for-PCR Kit; Clontech, Palo Alto, CA, USA). The PCR procedures for Ang-1 and Ang-2 were performed according to the protocol of Kim *et al* (2000a), while G3PDH (internal control) was provided in the RT-PCR kit. The sizes for the PCR product of Ang-1, Ang-2, and G3PDH were 1548, 1535, and 983 bp, respectively.

Immunoblot (Western blot)

In order to compare the differences in protein levels of the targets of interest (Ang-1, Ang-2, Tie-2, and VEGF) between pyogenic granulomas in pregnancy and those after parturition, a total of 10 gravidas with pyogenic granuloma and five non-pregnant, healthy young females were invited to join this study (mean age, 28 years; range, 20–35 years). The program was approved by the ethics committee of our hospital. Informed consent from all participants was secured.

As the granulomas in five gravidas hindered their normal occlusal function, they were excised in their second trimester of gestation. The remaining five available granulomas did not completely regress after parturition and were excised 2 weeks following parturition. Five normal gingival specimens from non-pregnant females were excised during crown lengthening procedure or gingivectomy and used as a control group. The granuloma or normal gingiva was hemisected for immunoblot and double staining. Half of the granuloma or normal gingiva was homogenized in a buffer solution (Tris chloride 50 mM, EGTA 2 mM, Dithiothreitol 2 mM, PMSF (phenylmethylsulfonyl fluoride) 0.5 mM, pH 8.0) for immunoblot. SDS-PAGE was carried out on gels of 12% acrylamide. Granuloma samples (80 μg protein for each lane) and 10 μl of molecular weight standards (New England Biolabs, Beverly, MA, USA) were applied to the gel. After electrophoresis, transferring, and blocking, the primary antibodies – polyclonal goat anti-human Ang-1 and Ang-2; polyclonal rabbit anti-human Tie-2 and VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) – were incubated with the membranes for 1 h at 37°C at a concentration of 1 μg ml^{-1}. The polyclonal rabbit anti-human β-actin (Santa Cruz Biotechnology) was used as an internal control for equal loading. After washes, secondary antibody with horseradish peroxidase (Santa Cruz Biotechnology) was incubated with the membrane for 1 h at 37°C at a concentration of 0.5 μg ml^{-1}. Following the final wash with PBST, the membranes were developed with Liquid DAB Black (Zymed Laboratories, San Francisco, CA, USA). The detected bands were scanned and analyzed by a scanning densitometer (Bio-Profil, Vilber Lourmat, Cedex, France) to compare the quantity.

Double staining (immunohistochemistry for Ang-2 plus TUNEL staining for apoptotic cells)

To understand the relationship between Ang-2 and cell apoptosis in pyogenic granuloma at different stages, we performed a double staining to detect the expression of Ang-2 and the extent of apoptosis on the same tissue specimens. Two kits (Histostain^{TM}-DS; Zymed Laboratories, Inc., San Francisco, CA, USA and TdT-FragEL; CN Biosciences, Inc., Cambridge, MA, USA) were used according to the manufacturers' instructions. Briefly, representative paraffin-embedded human granuloma sections on silane-coated slides were first deparaffinized and rehydrated. First, antibody against Ang-2 (Santa Cruz Biotechnology) in a concentration of 2 μg ml^{-1} was added and incubated at 4°C overnight. The following day, the slides were incubated with biotin-conjugated secondary antibody (Sigma Chemical Company, St Louis, MO, USA) at 37°C for 40 min after three PBS washes. Streptavidin-alkaline phosphatases were then applied at 37°C for 30 min after a PBS wash. NBT/BCIP (Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate) was used as chromogenic substrate for alkaline phosphatase and appeared dark blue. After three changes of PBS wash and the application of a double-staining enhancing agent, the second staining procedure (CN Biosciences) for apoptotic cells was based on indirect immunoperoxidase detection of biotin-labeled fragmented DNA. The positive reaction for terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) detection was a red coloration, which can be distinguished from the dark

blue coloration of Ang-2 staining. For quantification of the staining results, five fields of each slide were randomly selected and enumerated under ×400 magnification. The results of each slide were represented as the total number of apoptotic cells, and that of Ang-2 (+) blood vessel circles from five fields, respectively.

Three-dimensional tissue culture in fibrin gel

To directly examine the anti-angiogenic effect of adding Ang-2 and withdrawing VEGF, we performed a modified version of the Brown *et al* (1996) *in vitro* assay in fibrin gel. Briefly, a fragment of umbilical artery, approximately 2 mm in diameter and 3 mm in length, was excised from the umbilical cord within 48 h of a natural birth. Assays were performed in 24-well culture plates. Thirty microliters of thrombin (50 NIH U ml^{-1} in 0.15 M NaCl) (Sigma Chemical Company) was added to each well, followed by 1 ml per well of 3 mg ml^{-1} fibrinogen (Bovine plasma; Calbiochem-Novabiochem Co., La Jolla, CA, USA). The thrombin and fibrinogen were mixed rapidly, and one vessel fragment was quickly placed in the center of the well before clot formation. After gel formation, 1 ml per well of M199 medium with 10 ng ml^{-1} of recombinant human-VEGF (Bovine plasma; Calbiochem-Novabiochem Co.) was added. The vessel fragments were cultured at 37°C in a humidified 5% CO_2 environment for 5 days. Growth was observed by a phase-contrast microscope and quantified by determining the mean radius of microvessel outgrowths at both ends of the parent vessel in each culture well. On day 5, there was no significant difference between the mean radius values of microvessel outgrowth in each well. After PBS washes, the medium of each well was replaced with either 200 ng ml^{-1} bovine serum albumin (BSA; control), or 10 ng ml^{-1} rh-VEGF, or 200 ng ml^{-1} rh- Ang-2 (R&D Systems, Minneapolis, USA) or VEGF + Ang-2 in Hank's buffer salt solution (Gibco BRL®; Life Technologies, Rockville, MA, USA). The culture plates were incubated in the same humidified condition for another 5 days. At the end of the experiment, the plates were observed and photographed under a phase contrast microscope. The mean radius of microvessel outgrowth in each well was calculated under 100× magnification. Then the solution was discarded and fixed with 5% formalin for paraffin embedding and future TUNEL staining on apoptotic cells. The assays for each group were performed six times.

Statistical analysis

All data are presented as mean ± standard deviation (s.d.). Because of small sample sizes, statistical significance between groups in each assay was tested using the Kruskal–Wallis test, followed by the Wilcoxon signed-rank test. Statistical significance was set at $P < 0.05$. All statistical analyses were performed by StatView 4.5 software (Abacus Concepts, Berkeley, CA, USA).

Results

RT-PCR and Western blot

The sizes of PCR products from the experiments were correct, i.e. ~1.5 kb for Ang-2 and Ang-1, and ~1.0 kb for G3PDH. Two PCR products, one from each of the Ang-1 and Ang-2 experiments, were randomly chosen and sent for nucleic acid sequencing to confirm the correctness. Regarding the responses to the addition of rh-TNF-α after 6 h, all four endothelial cell types exhibited the same pattern. The expression of Ang-2 mRNA was upregulated by TNF-α in a dose-dependent manner from 0 to 50 ng ml^{-1}, while that of Ang-1 and G3PDH (internal control) remained unchanged. Because of the great similarity of the four cell types, only the result of HUAEC is presented (Figure 1). This result indicated that TNF-α (up to 50 ng ml^{-1}) could enhance the expression of Ang-2 by endothelial cells *in vitro*.

After quantifying the results of the Western blots, the expressions of Tie-2 and Ang-2 were significantly greater in the granulomas in pregnancy than in those after parturition. The levels of these two proteins were significantly higher in both stages of pyogenic granulomas than in the normal control. There was no significant difference between the expressions of Ang-1 and β-actin (internal control) in normal gingiva and in the two stages of pyogenic granulomas. VEGF can be detected significantly more in the granulomas in pregnancy than in those after delivery or in normal gingiva. There was no significant difference in the protein quantity of VEGF between normal gingiva and granuloma after parturition (Figure 2a and b). The results of Western blots suggested that VEGF in pyogenic granuloma after parturition decreased to a minimal level, similar to that found in normal gingiva. Ang-2 also decreased after delivery, although the level was still higher than that of normal gingiva, in which no active angiogenesis could be seen.

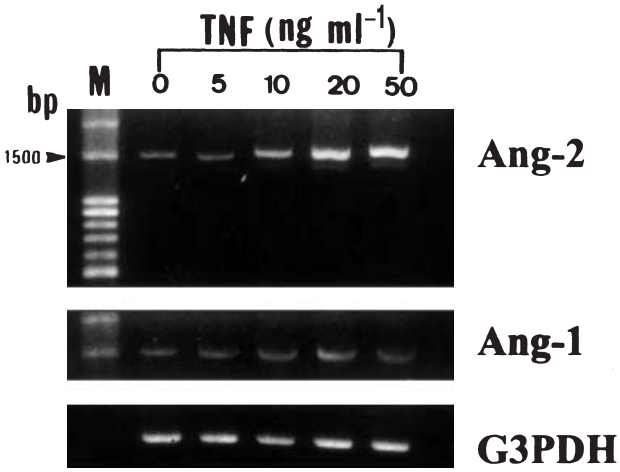

Figure 1 The results of reverse transcriptase-polymerase chain reaction of angiopoietin-2 (Ang-2) mRNA in endothelial cells are affected by tumor necrosis factor-α (TNF-α). As all four endothelial cell types [human umbilical vein endothelial cells, human umbilical artery endothelial cells (HUAEC), human saphenous vein endothelial cells, and human iliac artery endothelial cells] exhibited the same expression pattern, the result from HUAEC was taken to be representative. The mRNA of Ang-2 were upregulated by TNF-α from 0 to 50 ng ml^{-1}, while that of Ang-1 and G3PDH (internal control) remained unchanged

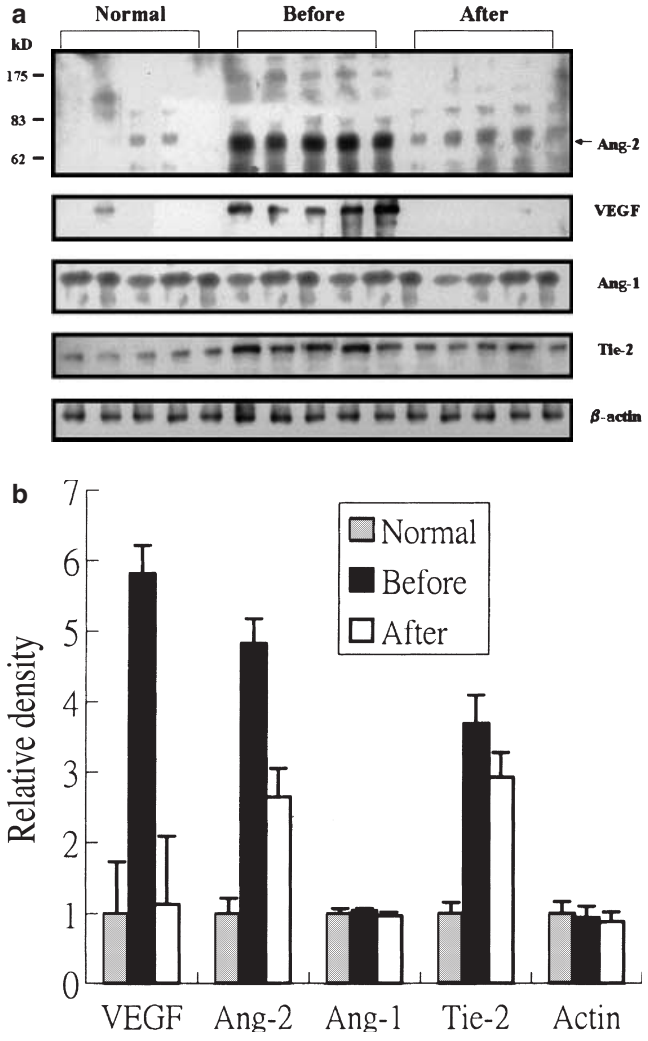

Figure 2 Western blot analyses of angiopoietin-2 (Ang-2), Ang-1, Tie-2, vascular endothelial growth factor (VEGF), and β-actin (internal control) on normal gingiva and pyogenic granulomas before and after parturition. (**a**) Five samples of each group were loaded (80 μg) in each lane and underwent immunoblot assays. The molecular weights for Ang-2, Ang-1, Tie-2, VEGF, and β-actin are ~70, 70, 120, 23, and 45 kD, respectively. Lane M depicts the protein markers. (**b**) Quantitative analysis by densitometer was presented as the relative ratio, which was normalized with the average density of tested protein bands from five samples of the normal gingiva group. Bars represent $\pm$s.d. ($n = 5$ for each group). A significant difference was found between granuloma in before- and after-parturition groups in the detection of VEGF, Ang-2, and Tie-2. A significant difference was found between granuloma in before- and after-parturition and normal gingiva groups in the detection of Ang-2 and Tie-2

Double staining and fibrin gel assay

The positive staining circles of Ang-2 in pyogenic granuloma in pregnancy and those after parturition were 23.6 $\pm$ 8.3 and 9.2 $\pm$ 4.0, respectively. A significant difference was found between these two groups. Ang-2 was almost undetectable in the normal gingiva group. Positive staining for Ang-2 was predominantly on endothelial cells and macrophage-like cells (Figure 3). The positive staining for apoptotic cells in granulomas in pregnancy and those after parturition were 11.5 $\pm$ 1.1 and 56.5 $\pm$ 19.8, respectively.

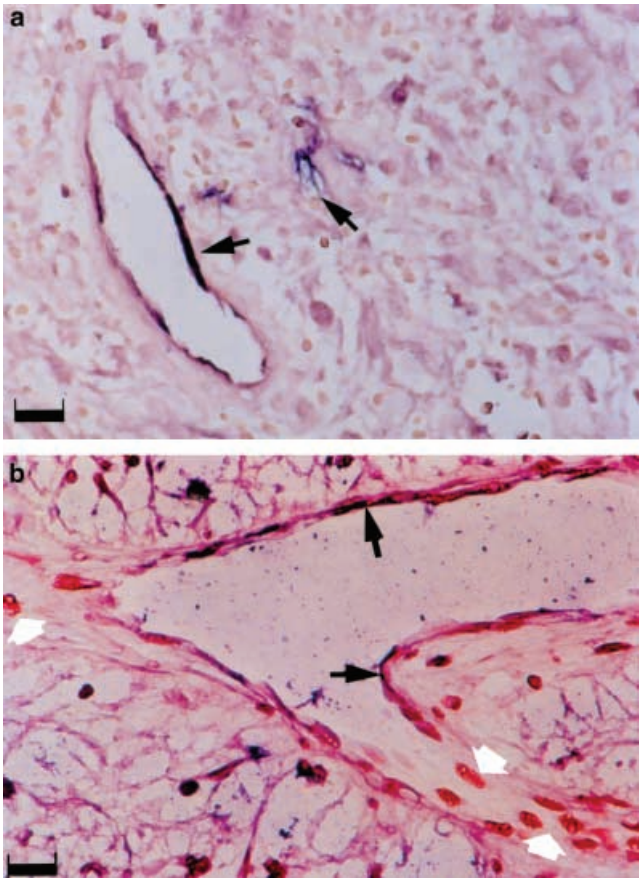

Figure 3 Double staining [immunohistochemistry for angiopoietin-2 (Ang-2) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling for apoptotic cells] in pyogenic granulomas before and after parturition. (**a**) Some endothelial cells were positive for Ang-2 expression (black arrows; positive reaction is indicated by a dark blue color) in a representative tissue section from granuloma during pregnancy. No significant apoptotic cell was seen. (**b**) A representative tissue section from granuloma after parturition. Ang-2 (black arrows) could also be detected on some endothelial cells. Positive staining for fragmented DNA (a sign of apoptosis) was detected mostly on smooth muscle cells (white arrows). Original magnification ×400; scale bar = 25 μm

Significantly more apoptotic cells could be seen in the sections of granulomas after parturition than those in pregnancy. Most of the apoptotic cells in granuloma after parturition were endothelial and smooth muscle cells (Figure 3). In the fibrin gel assay, the mean radius of microvessel outgrowth in BSA control, Ang-2, VEGF, and Ang-2/VEGF groups were 4.0 $\pm$ 4.1, 2.0 $\pm$ 2.7, 402.0 $\pm$ 37.0, and 390.0 $\pm$ 43.0 μm, respectively. No significant differences existed between the control and Ang-2 groups or between VEGF and Ang-2/VEGF groups. The two latter groups exhibited a significantly longer radius of microvessels than the two former groups. The numbers of apoptotic cells, after TUNEL staining in BSA control for Ang-2, VEGF, and Ang-2/VEGF groups, were 240.0 $\pm$ 58.2, 236.8 $\pm$ 67.2, 10.0 $\pm$ 4.2, and 10.8 $\pm$ 5.2, respectively. Similar to the result of microvessel outgrowth, a significant difference existed only between groups with and without VEGF. That is, fewer apoptotic cells could be detected in VEGF

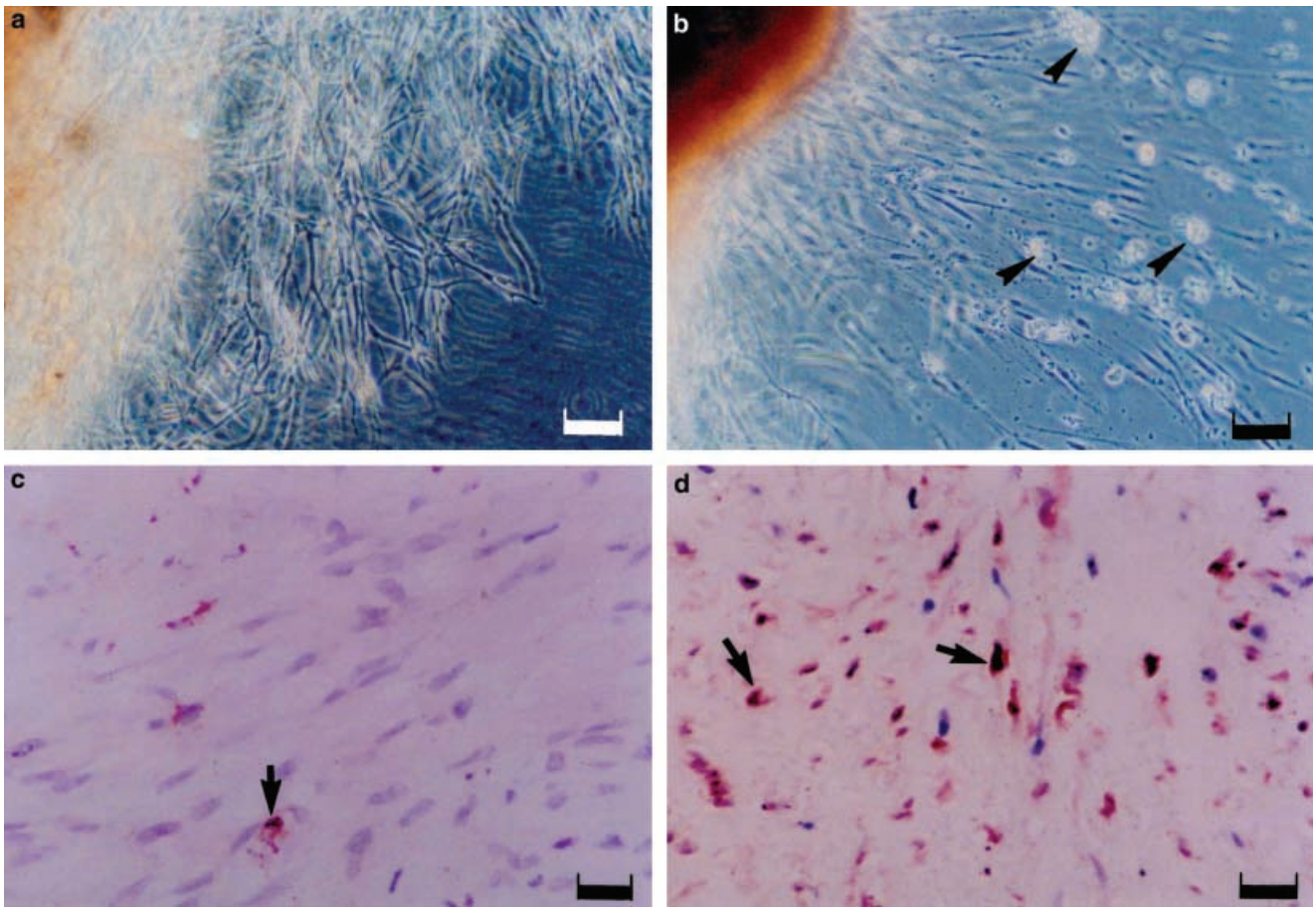

Figure 4 *In vitro* microvessel regression assay by tissue culture in fibrin gel. Because there were no significant differences between the bovine serum albumin and angiopoietin-2 (Ang-2)-alone groups, and between the vascular endothelial growth factor (VEGF)-alone and Ang-2 + VEGF groups, we present only two groups of results. (**a**, **b**) The microvessel outgrowths were significantly more abundant and healthy-looking in VEGF(+) groups (**a**) compared with VEGF(−) groups (**b**). Severe cell death (black arrow heads) was noted in VEGF(−) groups (phase-contrast microscope; ×100 magnification; scale bar = 100 μm). (**c**, **d**) Representative terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling staining for comparing apoptotic cells in fibrin gels with or without VEGF. Significantly less apoptosis (black arrows) was detected on VEGF(+) groups (**c**) than VEGF(−) groups (**d**). Original magnification ×400; scale bar = 25 μm

and Ang-2/VEGF groups than in Ang-2 groups alone or negative control groups (Figure 4). Both of the assays suggested that differences depended only on the existence of VEGF.

Discussion

In our previous study, we found angiogenic factors (VEGF, bFGF) were significantly lower in the pyogenic granuloma after parturition than those in pregnancy. In contrast, there was significantly more TNF-α in the granuloma after parturition (Yuan *et al*, 2002). As Ang-2 was regarded as an important regulator in the blood vessel regression and could be upregulated by TNF-α (Hanahan, 1997; Kim *et al*, 2000b), we were very interested to know whether a shortage of VEGF and Ang-2 elevated by TNF-α caused the natural regression of pyogenic granuloma after delivery. In the first part of the study, we found that TNF-α did upregulate the transcription of Ang-2 in a dose-dependent manner in all the tested endothelial cell types. This result indicated that upregulation of Ang-2 by TNF-α was general rather than specific among various types of endothelial cells.

In the Western blot assay, the levels of VEGF were significantly lower in the normal gingiva and granuloma after parturition than in those in pregnancy. This result was consistent with the ELISA assay results of our previous study. Unexpectedly, the amount of Ang-2 protein was significantly less in the granuloma after parturition than during pregnancy, although it was still higher than that of normal gingiva. This may be because TNF-α is not the only protein that can upregulate the expression of Ang-2. It has been reported that hypoxia, VEGF, bFGF, transforming growth factor-beta (TGF-β), and thrombin can also upregulate Ang-2 expression (Mandriota and Pepper, 1998; Oh *et al*, 1999; Huang *et al*, 2002; Scott *et al*, 2002). As we demonstrated before, VEGF and bFGF were abundant in the granuloma during pregnancy and minimal after parturition. Therefore, it is reasonable to conclude that, after parturition, despite increased TNF-α, there still appears

to be less downstream Ang-2 than during pregnancy due to decreased VEGF and bFGF. Furthermore, a recent study found that daily administration of 17-β-estradiol for 8 days in ovariectomized rats resulted in a significant increase in Ang-2 mRNA in non-reproductive tissues (Ye *et al*, 2002). This is also in accordance with our results because estrogen level is unquestionably much higher during pregnancy than after delivery. Our double staining results showed less Ang-2 and more apoptotic cells detected in the sections of granulomas after parturition than during pregnancy. This was consistent with the Western blot assay and our previous study (Yuan *et al*, 2002). More Ang-2 was not associated with more apoptosis of granuloma cells. In summarizing the results of Western blot and double staining, Ang-2 seemed not to be the determinant in the regression of pyogenic granuloma. Nonetheless, the findings did not argue against the molecular hypothesis for blood vessel regression proposed by Hanahan (1997), because VEGF had to be simultaneously taken into consideration.

In the *in vitro* fibrin gel assay, with fewer confounding effects from other cytokines than *in vivo*, Ang-2 did not exhibit a significant influence on the regression of newly grown microvessels. Ang-2 alone (in a concentration of 200 ng ml^{-1}) did not cause more apoptosis in vessel fragments than in the negative control. In combination with VEGF, Ang-2 did not induce more microvessel outgrowth than VEGF alone. Lack of proper interaction between endothelial cells and smooth muscle cells in this model (the microvessel outgrowths are purely endothelial cells), may be partly responsible for these inconclusive findings. VEGF at a concentration of 10 ng ml^{-1} in this model was enough to protect microvessels from apoptosis and regression, while severe apoptosis was noted in groups without VEGF. In granuloma after parturition, the levels of angiogenic factors (VEGF, bFGF) dropped below 10 ng g^{-1} of tissue. After losing the protection of VEGF or bFGF, the newly grown blood vessels in the granuloma start to undergo apoptosis and regress, as we can see clinically.

The next question is how VEGF diminished after parturition. Two plausible mechanisms may account for the results. First, the number of cells that secrete VEGF decreased. Secondly, the secretion of angiogenic factor was reduced. Leukocytes (especially macrophages) and endothelial cells are the major sources for angiogenic factors (Sunderkotter *et al*, 1994). Female sex hormones have been proven to have protective effects on endothelial cells and leukocytes (Spyridopoulos *et al*, 1997; Vegeto *et al*, 1999; Grimaldi *et al*, 2002). After parturition, while female sex hormones return to basal levels, granuloma cells may face a challenge from TNF-α and other apoptosis-inducing molecules (Hamacher *et al*, 2002). The cells undergo apoptosis without further protection. Furthermore, intervening (non-surgical or surgical) clinical treatments decrease or eradicate the inflammatory stimulus and therefore stop recruiting new leukocytes (Witko-Sarsat *et al*, 2000; Yuan *et al*, 2002). Consequently, the inflammatory cytokines (upstream of VEGF) gradually decrease through a vicious cycle of negative feedback, and the granuloma cells enter a default pathway of apoptosis without the protection from growth factors or female sex hormones.

Our study suggested that the lack of VEGF played an important role in the natural course of granuloma regression. This can provide new directions in developing non-surgical therapy (in combination with conventional debridement methods) for those gravidas suffering from pyogenic granulomas. Of all anti-angiogenesis therapies, two molecules (angiostatin and endostatin) especially deserve further investigation for possible applications (Sledge and Miller, 2002). Both are capable of downregulating VEGF *in vitro* and *in vivo* (Hajitou *et al*, 2002). Angiostatin markedly inhibits the migration of monocytes and neutrophils to inflammatory sites (Benelli *et al*, 2002). Interestingly, angiostatin inhibits pathological angiogenesis without affecting the development of physiological vascularization (Drixler *et al*, 2001). Recombinant human endostatin has entered phase I clinical study and has been proven safe and well-tolerated (Herbst *et al*, 2002). The time to employ innovative non-surgical treatment on gravidas with pyogenic granulomas may not be too far away.

Acknowledgements

This study was supported in part by grants NCKUH 90-056 from National Cheng Kung University Hospital and NSC 90-2314-B-006-127 from the National Science Council of the Republic of China.

References

Asahara T, Chen D, Takahashi T *et al* (1998). Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. *Circ Res* **83:** 233–240.

Benelli R, Morini M, Carrozzino F *et al* (2002). Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. *FASEB J* **16:** 267–269.

Brown KJ, Maynes SF, Bezos A *et al* (1996). A novel in vitro assay for human angiogenesis. *Lab Invest* **75:** 539–555.

Drixler TA, Rinkes IH, Ritchie ED *et al* (2001). Angiostatin inhibits pathological but not physiological retinal angiogenesis. *Invest Ophthalmol Vis Sci* **42:** 3325–3330.

Grimaldi CM, Cleary J, Dagtas AS *et al* (2002). Estrogen alters thresholds for B cell apoptosis and activation. *J Clin Invest* **109:** 1625–1633.

Hajitou A, Grignet-Debrus C, Devy L *et al* (2002). The antitumoral effect of endostatin and angiostatin is associated with a down-regulation of vascular endothelial growth factor expression in tumor cells. *FASEB J* **16:** 1802–1804.

Hamacher J, Lucas R, Lijnen HR *et al* (2002). Tumor necrosis factor-alpha and angiostatin are mediators of endothelial cytotoxicity in bronchoalveolar lavages of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* **166:** 651–656.

Hanahan D (1997). Signaling vascular morphogenesis and maintenance. *Science* **277:** 48–50.

Herbst RS, Hess KR, Tran HT *et al* (2002). Phase I study of recombinant human endostatin in patients with advanced solid tumors. *J Clin Oncol* **20:** 3792–3803.

Huang YQ, Li JJ, Hu L *et al* (2002). Thrombin induces increased expression and secretion of angiopoietin-2 from human umbilical vein endothelial cells. *Blood* **99:** 1646–1650.

Kim I, Kim JH, Moon SO *et al* (2000a). Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. *Oncogene* **19:** 4549–4552.

Kim I, Kim JH, Ryu YS *et al* (2000b). Tumor necrosis factor-alpha upregulates angiopoietin-2 in human umbilical vein endothelial cells. *Biochem Biophys Res Commun* **269:** 361–365.

Little JW (1988). Pregnancy and breast-feeding. In: Little JW, Falace DA, eds. *Dental management of the medically compromised patient*, 3rd edn. The CV Mosby Company: St Louis, pp. 325–331.

Maisonpierre PC, Suri C, Jones PF *et al* (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277:** 55–60.

Mandriota SJ, Pepper MS (1998). Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ Res* **83:** 852–859.

Oh H, Takagi H, Suzuma K *et al* (1999). Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J Biol Chem* **274:** 15732–15739.

Pacifici R, Brown C, Puscheck E *et al* (1991). Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. *Proc Natl Acad Sci USA* **88:** 5134–5138.

Scott BB, Zaratin PF, Colombo A *et al* (2002). Constitutive expression of angiopoietin-1 and -2 and modulation of their expression by inflammatory cytokines in rheumatoid arthritis synovial fibroblasts. *J Rheumatol* **29:** 230–239.

Seymour RA (1992). The sex hormones. In: Seymour RA, Heasman PA, (eds). *Drugs, diseases, and the periodontium*. Oxford University Press: New York, pp. 135–138.

Sledge GW Jr, Miller KD (2002). Angiogenesis and antiangiogenic therapy. *Curr Probl Cancer* **26:** 1–60. Review.

Spyridopoulos I, Sullivan AB, Kearney M *et al* (1997). Estrogen-receptor-mediated inhibition of human endothelial cell apoptosis. Estradiol as a survival factor. *Circulation* **95:** 1505–1514.

Sunderkotter C, Steinbrink K, Goebeler M *et al* (1994). Macrophages and angiogenesis. *J Leukoc Biol* **55:** 410–422. Review.

Tabibzadeh S, Satyaswaroop PG, von Wolff M *et al* (1999). Regulation of TNF-alpha mRNA expression in endometrial cells by TNF-alpha and by oestrogen withdrawal. *Mol Hum Reprod* **5:** 1141–1149.

Vegeto E, Pollio G, Pellicciari C *et al* (1999). Estrogen and progesterone induction of survival of monoblastoid cells undergoing TNF-alpha-induced apoptosis. *FASEB J* **13:** 793–803.

Witko-Sarsat V, Rieu P, Descamps-Latscha B *et al* (2000). Neutrophils: Molecules, functions and pathophysiological aspects. *Lab Invest* **80:** 617–653. Review.

Ye F, Florian M, Magder SA *et al* (2002). Regulation of angiopoietin and Tie-2 receptor expression in non-reproductive tissues by estrogen. *Steroids* **67:** 305–310.

Yuan K, Wing LY, Lin MT (2002). Pathogenetic roles of angiogenic factors in pyogenic granulomas in pregnancy are modulated by female sex hormones. *J Periodontol* **73:** 701–708.