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ANATOMICAL PATHOLOGY

Increased apoptosis and secretion of tryptase by mast cells in infantile haemangioma treated with propranolol

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Summary

Propranolol is increasingly used to treat problematic infantile haemangioma (IH), although its molecular mechanisms remain unclear. A key feature of propranolol therapy is the decreased deposition of fibrofatty residuum compared with spontaneously involuting IH. This study investigated the molecular consequences of propranolol treatment for IH *in vivo*. Immunohistochemical and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining was performed on five age matched patients with proliferative IH. Two patients (A and B) were undergoing propranolol treatment at the time of surgical resection. Propranolol treatment increased apoptosis, and induced mast cells to degranulate and secrete tryptase into the interstitium. The microvessels of patient A were immature [weak von Willibrand Factor (vWF), and strong osteoprotegerin (OPG) staining], comparable to untreated proliferative IH, while those of patient B were mature (strong vWF staining, and no OPG staining). The perivascular $CD90^+$ mesenchymal stem cell population was preserved in both propranolol treated patients. Using rarely obtained biopsies from IH patients treated with propranolol, we show increased apoptosis by propranolol for the first time *in vivo*. We also suggest that mast cells, through secreted proteases, may contribute to the decreased fibrofatty residuum seen with propranolol treatment.

Key words: Apoptosis, endothelial cells, immunohistochemistry, infantile haemangioma, mast cells, mesenchymal stem cell, microvessel maturity, osteoprotegerin (OPG), propranolol, tryptase.

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INTRODUCTION

Infantile haemangioma (IH) is a common microvascular tumour of infancy. IH is most common in Caucasian, female, and premature infants, affecting 5–10% of the population. Three phases of growth characterise the progression of IH, lasting up to 12 years of age. In proliferative IH (up to 12 months of age), pericytes and plump endothelial cells (ECs) with scattered mitotic figures form small tightly packed capillaries. These capillaries are immunoreactive for EC markers CD31 and CD34, as well as the definitive IH marker GLUT1. As these lesions involute, there is a thickening and lamination of the capillary basement membrane, a flattening of the ECs, and increased deposition of fibrous stroma between the vessels. Large vessels separated by a sparse fibrofatty residuum characterise the fully involuted lesion.[1–4]

In cases of IH involving significant morbidity, corticosteroids or surgery have been the preferred treatment options.[5,6] Recently the non-specific β-blocker propranolol has emerged as an effective and non-invasive therapy eliciting a rapid regression of the lesion with a significantly reduced fibrofatty residuum.[5,7,8] Despite its therapeutic success, the underlying molecular mechanisms of propranolol action on IH are not fully understood. Proposed mechanisms include pro-apoptotic, anti-angiogenic and vasoconstrictive effects,[9] as well as modulation of the renin angiotensin system (RAS).[10] The elucidation of propranolol's mechanism remains difficult because of the scarcity of propranolol treated IH tissue for analysis.

Additional actions of propranolol treatment may be possible. Acceleration of EC maturation normally seen during IH involution,[1,4] in which osteoprotegerin (OPG) is lost from and strong von Willibrand Factor (vWF) staining is gained by ECs,[11] represents a possible mechanism of action. A significant benefit of propranolol treatment is the reduced fibrofatty residuum. This residuum is possibly derived from the perivascular mesenchymal stem cell (MSC) population,[12–14] the regulation of which has been proposed as a therapeutic target for IH.[8] Whether this population is reduced by propranolol treatment is unknown. Additionally, mast cells synthesise and secrete a range of bioactive mediators, including proteases that degrade the extracellular matrix (ECM). Their abundance increases during involution, suggesting a role for mast cells in the resolution of this lesion.[15] Whether mast cells play a role in the action of propranolol therapy is unexplored.

Increased apoptosis, accelerated microvessel maturation and degradation of the ECM by mast cells may be important components of the mechanism of propranolol action. Here we examine these molecular components in biopsies from two propranolol treated patients whom later also underwent surgical resection.

MATERIALS AND METHODS

Patients

Tissue collection and processing were approved by the Wellington Regional Ethics Committee, and signed consent for the use of tissue was obtained from the patients' next-of-kin. Solid biopsies were collected from the centre of the lesions, often including subcutaneous tissue, of five patients with proliferative IH aged between 4 and 8 months (mean 6 months) at the time of surgical resection. Biopsies were immediately fixed in 10% formalin overnight then paraffin embedded using standard protocols for long-term storage at room temperature. Samples stored in paraffin are stable for several years, although typically tissue used was less than 1 year old. Of these five patients, two (A and B) were treated with propranolol (mean 5 months at age of biopsy) while the remaining three (mean 6 months) received no treatment. The diagnosis of IH for all patients was made by a trained pathologist based on the histopathology of the lesions, as well as expression of the definitive IH marker, GLUT1.[4]

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Patient A

Patient A presented with an ulcerating IH of the right earlobe at 2.5 months of age and treatment with 1.5 mg/kg/day propranolol was started. The patient experienced on-going ulceration and pain despite slow regression of the haemangioma, so the lesion was debulked at 4 months of age. Following surgery, propranolol treatment was stopped, during which time the haemangioma increased in size. Propranolol treatment was reinstated 3 weeks after surgery at 2 mg/kg/day, which resulted in continued regression of the lesion.

Patient B

Patient B presented with an ulcerating haemangioma on the buttock at 4 months of age and treatment with 1.5 mg/kg/day propranolol was started. Slower than expected regression and ongoing ulceration was observed. At 6 months of age a biopsy was taken, but the majority of the haemangioma was left *in situ*. The patient remained on propranolol treatment and the lesion continued to gradually regress.

Immunohistochemistry

Immunohistochemistry was performed on 4 μm paraffin sections as previously described,[11,13,16,17] using primary antibodies raised in either rabbit or mouse against CD34 (1:300; Invitrogen, USA), CD31 (1:300; Abcam, UK), OPG (1:100; R&D Systems, USA), tryptase (1:500; Abcam), von Willibrand Factor (vWF; 1:200; Abcam), CD90 (1:200; Abcam) and smooth muscle actin (SMA; 1:200; Abcam). Species-specific secondary antibodies conjugated to either Alexa Flour 488 or 555 (1:500; Invitrogen) were used to visualise epitope bound primary antibodies. Slides were mounted in ProLong Gold Antifade containing 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen) to visualise cell nuclei.

Apoptosis detection

Apoptotic nuclei were detected using an In Situ Cell Death Detection Kit (Roche, Switzerland) following the manufacturer's instructions. This kit is based on the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) technique, in which fluorescein-labelled nucleotides are attached at sites of single and double stranded DNA breaks typical of apoptotic cells. The TUNEL reaction was performed after the washing of primary antibodies, and before secondary antibody incubation.

Image acquisition and statistical analysis

Quantitative analyses were performed on the frequency of TUNEL stained nuclei, and on the maturity of the microvessels within IH on the basis of OPG and vWF staining.[11] Multiple fields of view (typically 5 fields for OPG/vWF stained sections, and 25 fields for TUNEL stained sections) were obtained for these analyses to ensure that at least 100–200 events were counted per patient. The number of apoptotic nuclei per field of view was expressed as the percent of all nuclei, whereas the number of immature OPG positive vessels was expressed as the percent of all vessels with an identifiable lumen. All images were captured using an Olympus FV1000 confocal laser-scanning microscope fitted with a krypton/argon laser (Olympus, Japan), and a Student's *t*-test was used to compare group means.

RESULTS

Effective therapy had been implemented in both patients treated with propranolol, although the rate of regression was slower than expected. The lesions of both patients were histologically proliferative containing microvessels that were immunoreactive for the definitive IH marker, GLUT1 (Fig. 1).[4] Thus, our analyses report effects of propranolol at the earliest stages of regression.

Apoptosis

Enhanced apoptosis has been suggested as a possible mechanism for accelerated involution of IH by propranolol.[9] Figure 2 shows TUNEL-labelled (green) apoptotic nuclei (counterstained with DAPI, blue) in both propranolol treated and untreated IH. Figure 2A shows a low level of apoptotic nuclei (yellow arrowheads) in untreated patients, while Fig. 2B shows many more TUNEL labelled nuclei in propranolol treated patients. The percentage of TUNEL reactive nuclei in both propranolol treated patients was significantly greater than that observed for untreated patients (Fig. 2C, $p < 0.05$).

Microvessel maturation

We have previously shown that OPG expression by immature microvessels is replaced by vWF expression as they mature.[11] Strong OPG expression was seen in 89% of the microvessels of untreated patients (green), and these vessels expressed only low levels of vWF (red, Fig. 3A). In propranolol treated tissue, patient A was indistinguishable from the control group (data not shown). In patient B, however, very few of the microvessels were OPG positive and most vessels were also strongly vWF immunoreactive (Fig. 3B). Figure 3C graphically shows the fraction of vessels that were OPG positive for the untreated IH ($n = 3$), as well as each of the propranolol treated patients (patient A and patient B).

Tryptase secretion by mast cells

The presence of tryptase positive mast cells in IH has been previously reported.[18] Figure 4 identifies mast cells by way of tryptase immunoreactivity (green staining) in untreated (Fig. 4A) and a propranolol treated IH (Fig. 4B). The endothelium is identified by staining for CD31 (red), while nuclei are stained with DAPI (blue). Figure 4B shows that in propranolol treated tissue the mast cells have degranulated and secreted copious amounts of tryptase into the interstitium, while very little interstitial tryptase immunoreactivity was detected in any of the untreated IH patients (Fig. 4A). Increased interstitial tryptase was detected in both propranolol treated patients A and B.

DISCUSSION

Propranolol has become the preferred treatment for IH with significant morbidity. Patients treated with propranolol

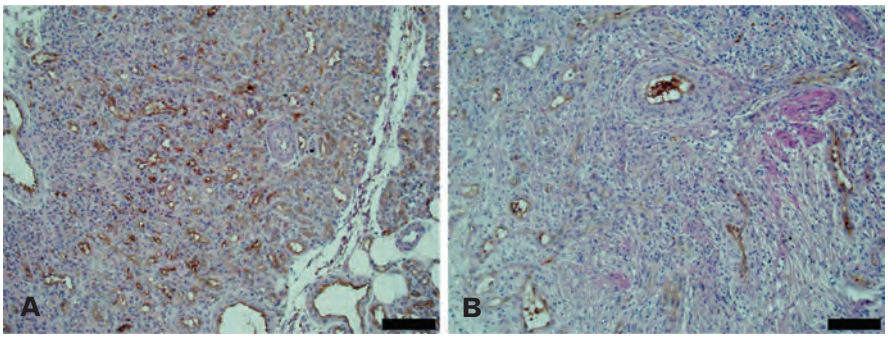

Fig. 1 Confirmation that propranolol treated tissues represent proliferative IH. Histological analysis by H&E and DAB staining showed tightly packed endothelial cells and pericytes forming microvessels expressing GLUT1 (appears brown) in both propranolol treated patient A (A) and patient B (B), confirming the diagnosis of IH. Scale bar = 100 μm.