ORIGINAL RESEARCH

Visualisation of human dental pulp vasculature by immunohistochemical and immunofluorescent detection of CD34: A comparative study

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Abstract

CD34 is considered a pan-endothelial cell marker for paraffin-embedded sections. In this study, both immunohistochemistry and immunofluorescence were applied in human dental pulp specimens of moderate thickness (10 µm) in order to observe the vasculature of this tissue using CD34. Both techniques revealed a homogenous staining pattern with capillaries and larger vessels showing complete and strong membrane staining reflecting the high capacity of the pulp for regeneration and response to different stimuli. A novel approach in the identification of the pulpal vasculature by Cy5-conjugated anti-CD34 is introduced in this study. By this technique the dense capillary plexus of the sub-odontoblastic region, which is responsible for the reaction of the tissue to any physical or chemical stimuli or pathological condition, can be clearly identified, while immunohistochemistry did not reveal such a detailed staining pattern.

Introduction

Dental pulp is a unique soft tissue that includes highly specialised cells including odontoblasts, fibroblasts, antigen-presenting cells, immune-cells, connective tissue fibres, a large number of blood vessels and a broad dense plexus of nerve fibres and bundles. Regarding its vascular plexus structure, dental pulp is restricted during inflammation, as no ability of a parallel vascularity plexus exists, owing to its isolation within the solid dentinal walls of the pulp chamber.

The entire vascular system of the dental pulp comprises endothelial cells (ECs). A number of antigens within these cells have proved to be useful for the histological investigation of vascular structures. CD34 is an endothelial marker that is extensively used in immunohistochemistry (IHC) and has been recently used in dental pulp for the identification of pulpal vasculature (1).

CD34 is a cell surface protein that is expressed selectively by haematopoietic progenitor cells (HCs) and ECs. It is a heavily glycosylated Type I trans-membrane protein (2,3) with an intracellular domain containing consensus sites for a variety of kinases (4), and serves as a ligand for L-selectin in ECs (5). Both HCs and ECs come from a common progenitor, the haemangioblast, a transient cell stage that develops early and disappears quickly during embryonic development (6). Various endothelial markers have been used in order to identify the antigen reactivity of vessels in a variety of tissues (7–10).

According to Vermeulen *et al.* (11) and Hasan *et al.* (12), CD34 is considered a pan-endothelial marker for paraffin-embedded sections. When using CD34, the dental pulp vessel endothelium is easily identified under a confocal fluorescence microscope. However, under a light microscope (LM), the endothelium is not easily distinguished.

Therefore, in the present comparative study, the expression of CD34 in human dental pulp by applying light microscopic IHC and confocal microscopic immunofluorescence (IF) in paraffin-embedded tooth pulps was investigated. In this comparative study, a novel approach in identifying the microvasculature of human dental pulp is being introduced using Cy5-conjugated anti-CD34 and immunofluorescent confocal microscopy in specimens of moderate thickness (10 μm).

Materials and methods

Tissue specimens

The investigation was approved by the Ethical Committee of the Aristotle University of Thessaloniki, Greece. Fifteen premolar teeth from young patients (range 16–18 years), free of caries and restorations, were extracted owing to orthodontic reasons. The teeth were initially fixed in 10% formalin solution for 48 h. Pulps were gently removed and placed in the same fixative for 48 h. The specimens were embedded in paraffin blocks and 300 to 500 serial sections of 10 μm were obtained using a microtome HM340 E (MICROM Laborgeräte, Walldorf, Germany). From the above specimens 10 were treated with immunohistochemical methods and five with IF methods. Next, the sections were examined under LM or confocal microscope consequently.

Antibodies source

The EC marker (CD34) mouse monoclonal antibody (clone QBEnd/10) was purchased from Novocastra (Newcastle, UK). This antibody selectively detects human CD34 antigen in formalin-fixed and paraffin-embedded tissue. The LSAB+ Kit was purchased from DAKO (Glostrup, Denmark). Cy™5-conjugated goat anti-mouse antibody was purchased from Jackson (West Grove, PA, USA).

Light microscopic immunohistochemistry

The Avidin : Biotinylated enzyme complex (ABC) technique was used for the immunohistochemical method. The slides were deparaffinised in graded solutions of xylene, and dehydrated in graded ethanol series. Endogenous peroxydase was inhibited using 1.6% hydrogen peroxide in methanol. The sections were then washed in distilled water and heated in a microwave oven (in a citrate buffer 10 mM pH 6) for 21 min in order to achieve epitope retrieval. After rinsing with Tris-buffered saline (TBS) (DAKO) the slides were incubated with rabbit serum (DAKO) in TBS 1/5 for 20 min, and afterwards with the mouse monoclonal primary antibody CD34 (Novocastra) in 1/20 dilution overnight. Slides were again rinsed

with phosphate-buffered saline and then incubated with Biotinylated Rabbit Anti-Mouse immunoglobulins (DAKO) in 1/200 dilution for 30 min. The samples were washed with TBS and incubated with StrepABComplex/HRP (DAKO) at 1/100 dilution for 30 min. After one more wash with TBS the colour was developed with the chromogen 3, 3-diaminobenzidine (DAKO) for 5 min, followed by TBS washing and nuclear counterstaining with Harris haematoxylin for 45 s. Normal mouse serum replaced the primary antibody in negative control sections. Observation of the sections was conducted using a Zeiss LM (Axiostar, Zeiss, Wetzlar, Germany). The regions of interest from each section were digitised by a video camera, which was connected to the LM and to a computer video grabber.

Laser scanning confocal microscopy – immunofluorescence

Paraffin sections of 10 μm were deparaffinised and pretreated with 2.73% hydrogen peroxide and 0.1% sodium azide diluted with distilled water. Trypsin digestion was performed to all specimens for 30 s at 36°C. Slides were at first incubated with primary mouse CD34 monoclonal antibody diluted at 1/20 with TBS containing 5% fetal calf serum for 12 h at 4°C. Consequently, incubation of specimen with Cy™5-conjugated secondary antibody diluted at 1/100 with phosphate-buffered saline was performed for 12 h at 4°C. The slides were mounted in Mowiol and observed in a Leica TCS SP2 AOBS confocal laser scanning microscope using 20x oil immersion objective (HC PL APO CS, NA = 0.70). For Cy-5 detection, excitation with helium-neon laser at a wavelength of 633 nm and emission at 649–732 nm was applied in one channel (red), while in the second channel (green), the autofluorescence images were acquired at the excitation wavelength of 514 nm and detection range 572–709 nm. Sequential scanning was applied to avoid bleed-through from autofluorescence during Cy-5 detection. Slides without primary/secondary antibody were used as negative controls.

Statistical analysis

The number of vessels in each image was identified by three independent observers and the data were analysed using the statistics toolbox provided with MATLAB® (Mathworks, Natick, MA, USA). The mean difference in number of vessels between corresponding IF and IHC section pairs was calculated and the Wilcoxon signed rank test was used to test the significance of that difference. Also, the ratio of the mean number of vessels in IF to mean number of vessels in IHC sections was calculated. The significance of the ratio was tested by performing bootstrap

analysis, where 10 000 random samples of size 50 were generated, by sampling from the original 50 images with replacement, and then calculating the smallest mean ratio of the number of vessels in all these samples.

Analysis on the repeatability of the observations was also performed. For each section, the mean absolute deviation as the absolute difference of each observation from the mean of the observations was calculated. The mean absolute deviation was further divided by the mean of the observations, producing the fractional mean absolute deviation (FMAD). Statistical significance was established via the *t*-test, as well as bootstrap analysis, where 10 000 random samples of size 50 were generated, by sampling from the original 50 images with replacement, and calculating the percentage of samples where the mean FMAD for IF was smaller than the mean FMAD for IHC.

Results

Immunohistochemistry and light microscopy

The entire image of the dental pulp retained the appearance of loose connective tissue. Vascular endothelium of the dental pulp showed intense positive staining for CD34 by the ABC method. The capillaries of the sub-odontoblastic plexus were strongly positive for CD34, while odontoblasts were negative (Fig. 1a). The ECs of all types of blood vessels, ranging from capillaries (Fig. 1a) to arterioles and venules (Fig. 1b), were positive, exhibiting a homogenous and intense labeling pattern with a characteristic brown color. Fibroblasts and nerve bundles were negative (Fig. 1b). The background was clean.

Immunofluorescence and laser scanning confocal microscopy

Dental pulp vasculature demonstrated strong positive staining using Cy5-conjugated anti-CD34. ECs of capillaries and small vessels showed intense staining in all specimens that were investigated including the sub-odontoblastic region (Fig. 2a) and revealing a homogenous staining pattern. A high density of capillaries was observed in the sub-odontoblastic region (Fig. 2a). Odontoblasts and nerve bundles (Fig. 2a) were negative. The ECs of larger pulp vessels were positive as well (Fig. 2b), exhibiting a thin-linear staining, while thick and intense staining was observed on the capillaries' endothelium (Fig. 1a).

Statistical comparison of IF versus IHC methods

The mean difference in number of vessels between corresponding IF and IHC section pairs was 37.68, with a standard deviation of 13.72 and a *P*-value for the Wilcoxon signed rank test of 7.55×10^{-10}. The mean ratio of the mean number of vessels in IF to the mean number of vessels in IHC sections for all 50 section pairs was 3.32 with a standard deviation of 1.22. The histogram of this ratio (Fig. 3) shows a clear superiority for the IF method, with no cases where the ratio is smaller than unity, and a peak between 2 and 3. In the bootstrap analysis, the smallest mean ratio of number of vessels in corresponding IF and IHC sections in any of these samples was 2.82.

The mean FMAD over all IF sections was 0.059, versus 0.101 for the IHC sections. The histogram of the FMAD for the original samples (Fig. 4) also shows a clear superiority of the IF method. FMAD for IF sections is concentrated between 0 and 0.05, while FMAD for IHC sections spreads to larger values. For the *t*-test, the *P*-value is 0.0028, while for the bootstrap analysis, the percentage of samples where the mean FMAD for IF was smaller than the mean FMAD for IHC was 99.84%.

Discussion

Dental pulp is a metabolically active tissue with a high capacity for regeneration in response to different stimuli
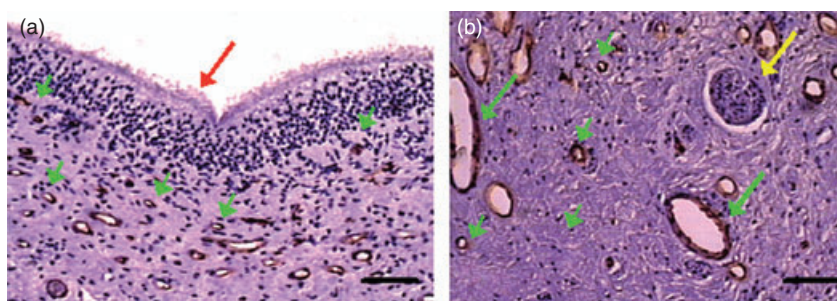

Figure 1 CD34 expression in human dental pulp vasculature investigated by immunohistochemistry in formalin-fixed and paraffin-embedded specimens (10 μm). The capillaries of the sub-odontoblastic plexus revealed positive staining to QBEnd10 by the Avidin : Biotinylated enzyme complex method (small green arrows), as well as larger vessels (large green arrows) (a,b). Odontoblasts (red arrow) (a) and nerve bundles (yellow arrow) (b) were negative. Bars: a, 100 μm; b, 50 μm.
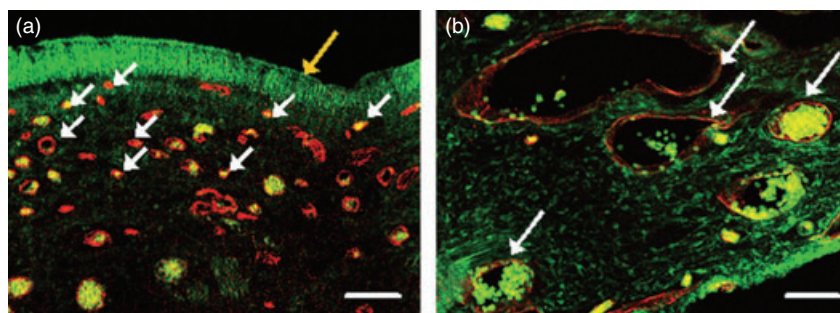
Figure 2 CD34 expression in human dental pulp vasculature investigated by immunofluorescence in formalin-fixed and paraffin-embedded specimens (10 μm). Intense staining of sub-ododntoblastic capillary plexus by Cy5-conjugated anti-CD34 in the red channel (small white arrows), while odontoblasts were negative (green channel) (yellow arrow) (a). Note the high density of the capillary plexus and some erythrocytes (yellow channel) within the small capillaries (a). Strong and homogenous staining pattern of the endothelial cells of the walls of the larger vessels (large white arrows), some full of erythrocytes (b). Bars: a, 100 μm; b, 50 μm.
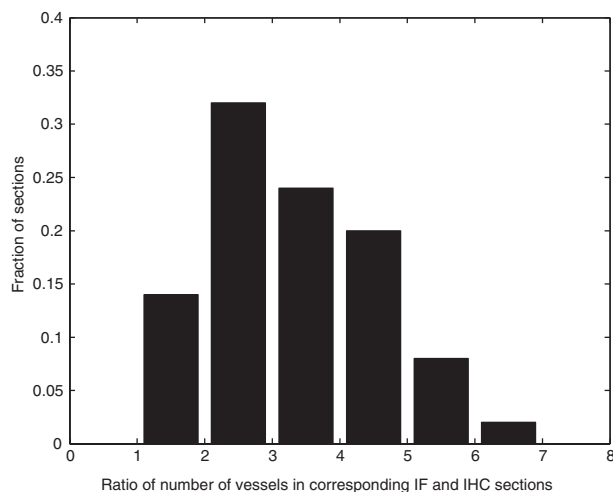

Figure 3 Histogram of the ratio of number of vessels detected on average by the three independent observers on all corresponding immunofluorescence (IF) and immunohistochemistry (IHC) images. The ratio is greater than unity for all 50 sections, with a peak between 2 and 3 and a mean of 3.32, indicating superiority of the IF method.
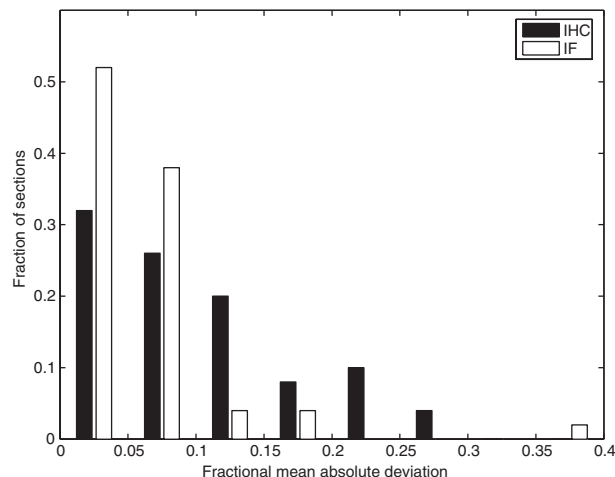

Figure 4 Histogram of the fractional mean absolute deviation (FMAD) of the observations of the three independent observers, for all immunofluorescence (IF) and immunohistochemistry (IHC) sections. FMAD for IF sections is concentrated between 0 and 0.05, while for IHC sections FMAD spreads to larger values. The mean for IF is 0.059 versus 0.101 for IHC, indicating greater repeatability for the IF method.

(13). ECs comprising the entire vascular network of the pulp are dynamic and capable of conducting a variety of metabolic and synthetic functions. This complex interplay of adaptation, development and maintenance of the vascular system requires a high degree of control and CD34+ cells of dental vascular endothelium have recently been shown to play a major role in tooth homeostasis, not only in embryonic development but also in the early stages of adult life (1).

The vascular endothelium is generally versatile and multifunctional, having many synthetic and metabolic properties. These include the regulation of thrombosis and thrombolysis, platelet adherence, modulation of vascular

tone and blood flow, and regulation of immune and inflammatory responses by controlling leukocyte, monocyte and lymphocyte interactions with the vessel walls (14). ECs play a major role in angiogenesis and vasculogenesis (15).

IHC in our study revealed a strong and homogenous staining pattern of capillaries as well as larger vessels (Fig. 1a,c). These results are in accordance with Trubiani *et al.* (1) who showed the presence of well-defined microvessels in the dental pulp of young adults, recognisable from the intense expression of CD34 antigen on EC surfaces, thus indicating the remodeling and vasculogenesis ability of dental pulp vessels. According to Nagatsuka

et al. (16), tumoral blood vessels positive for CD31, CD34 and CD105 are considered to represent neovessels with strong remodeling activity. The fact that capillaries of the sub-odontoblastic layer, which is the part of the dental pulp responsible for the reaction of the tissue to any physical or chemical stimuli and pathological condition, are intensely stained with CD34, adds to the above assumption. Cy5 conjugated to QBEnd10 revealed a homogenous and intense staining of capillary ECs in all levels of pulpal vasculature. A recent comparative study of EC markers expressed in chronically inflamed human tissues such as in rheumatoid arthritis, osteoarthritis and in Crohn's disease revealed a relative homogenous staining pattern of CD34 when examined by IF (17) reflecting the important role of blood vessels in tissue pathophysiology.

In our study, the capillaries of the sub-odontoblastic region determined by IF showed a remarkable density (Fig. 2a), indicating an advantage of IF method over the standard immuno-staining by the ABC technique. We have also shown IF to be more sensitive than the ABC method for immuno-staining within the vasculature of the sub-odontoblastic region. These results are in accordance with a previous comparative evaluation of these immuno-staining methods in rat tooth organs (18). Our results were further reinforced by statistical analysis methods, showing significantly higher vessel identification (mean vessel ratio in IF over IHC of 3.32) and repeatability (FMAD in IF of 0.059 versus 0.101 in IHC) for IF over IHC method. One possible explanation for this is that the staining procedure followed in IF allows better penetration of the antibodies and the colour products that are formed are more finely dispersed into the collagen-rich tissue of dental pulp. In line with this hypothesis is the fact that Cy5 is a small organic molecule conjugated to the secondary antibody in contrast to the large ABC complex. Additionally, these results may be explained in terms of staining intensity and least background staining, where Cy5 detection in the red channel of excitation is contrasted by a black background, while less contrast exists in the ABC method, with brown CD34 positive staining in a relatively dark purple background.

From the results of the present study, it can be concluded IF by Cy5 conjugated to QBEnd10 provides deeper insight into pulpal microvasculature compared with standard immuno-staining by the ABC technique.

Acknowledgements

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