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Stromal phenotype of dental follicle stem cells

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1. ABSTRACT

It has been suggested that stem/progenitor cells exist in dental tissue. This study identified adult mesenchymal stem/stromal cell-like populations in the dental follicle of human impacted third molars.The immunohistochemical analysis, of dental follicle using known stem-cell markers: Cytokeratins (AE1-AE3), Smooth Muscle Actin, Ki-67, CD34, CD44, CD45, CD56, and CD133. A positive reaction for at least one of the markers typical of stromal phenotype (CD56, CD44 and CD271) was observed in seven cases . Interestingly, all positive cases showed coexpression of CD44 and CD56, except for one case which was CD56 positive and CD44 negative. Immunohistochemical reaction was negative in all 27 cases for Ki-67, Cytokeratins, Smooth Muscle Actin, CD34, CD133 and CD45. The association: negative for CD34, CD45, CD133, and positive for CD44, CD56 (markers of a subpopulation of stem cells from bone marrow) suggests these may be quiescent mesenchymal stem cells, a hypothesis supported by the negativity of Ki-67 (proliferative index). Our results are compatible with the identification of immature fibroblast cells with phenotypic features of stromal stem cells in the dental follicle.

2. INTRODUCTION

Stem cell biology has become an important field for the understanding of tissue regeneration and for implementing regenerative medicine (1-3). Over recent decades, the search for Mesenchymal Stem Cells (MSC) in specific tissues has led to the discovery of a variety of stem cells in all of the body's organs and tissues (4,5). Dental-tissue derived MSC-like populations are among many other stem cells residing in specialized tissues that have been isolated and characterized (6-8). The first type of dental stem cell was isolated from the human dental pulp tissue and termed 'postnatal dental pulp stem cells' (DPSCs) (9). Three further types of dental-MSC-like populations were later isolated and characterized: stem cells from exfoliated deciduous teeth (SHED) (10), periodontal ligament stem cells (PDLSCs) (11), and stem cells from apical papilla (SCAP) (12). Further studies have identified a fifth dental-tissue-derived progenitor cell population, referred to as 'dental follicle precursor cells' (DFPCs) (13,14).

The dental follicle is a fibrous tissue surrounding the tooth germ, which can easily be isolated after wisdom-tooth extraction. It is an ectomesenchymal tissue composed of various cell populations derived from the cranial neural

crest, and playing a crucial role in tooth development (15). It harbors progenitor cells that differentiate during tooth development into cementoblasts, periodontal ligament fibroblasts and alveolar bone osteoblasts; these cells surround and support the teeth. The differentiation and function of dental follicular cells are controlled by a network of regulatory molecules, including growth factors and cytokines (16). It has additionally been shown that dental follicle precursor cells can differentiate into many different cell types, such as adipocytes and chondrocytes (17,18). Interestingly, neural-crest-derived dental follicle precursor cells and neural stem cells have a common ontogeny, and both can successfully differentiate into neurons (19). Dental stem cells, or precursor cells such as dental follicle cells, are easily accessible and comprise an excellent source of somatic stem cells (20,21).

Most studies addressing the identification of stem cells have been carried out *in vitro* (22-25): it has

been observed that dental follicle cells show a typical fibroblast-like morphology and express markers such as Notch-1 and Nestin, collagen type I, bone sialoprotein, osteocalcin, and fibroblast growth factor receptor 1-IIIC (13), suggesting the presence of undifferentiated cells. It has been reported that STRO-1 and bone marrow precursor receptors (BMPR) are expressed in dental follicles *in vivo* (26). The present study aimed to detect stem cells in dental follicles through immunohistochemical methods. Particularly, the study investigated the presence of progenitor cells in dental follicles from human third molar teeth by immunohistochemical staining for selected cell surface markers of mesenchymal stem cells.

3. MATERIALS AND METHODS

3.1. Patients and Design

Twenty-seven patients (8 m; 19 f) who were undergoing orthodontic therapy, having impacted third molars fully covered by mucosa, were included in the study; age-range was 15 to 25 years (mean, 18.7). Exclusion criteria included a history or sign of infection, and enlarged tissues surrounding impacted third molars. All patients signed a statement of informed consent to treatment, and material discarded or left over from pathology examinations was used for this study. All surgical treatment was carried out under local anesthesia by conventional third-molar surgery. Dental follicles (DFs) of impacted teeth were carefully curetted and separated from the mineralized tooth. The surfaces of the follicle tissues were cleansed using a sterilized scalpel. DFs were fixed in 10% buffered formalin and embedded in paraffin wax, sections (4 µm thick) were prepared and stained with hematoxylin and eosin (H&E) and Masson's trichrome. Additional slides were subjected to immunohistochemical staining. All specimens were examined by two pathologists. The criteria for classifying DF as histologically normal were: presence of fibrous connective tissue with remnants of reduced enamelepithelium in the fibrous connective tissue layer; absence of epithelial lining. Two components of DFs, epithelial and mesenchymal, were examined histologically. Histologic characteristics of epithelial and mesenchymal tissues were then correlated with age and gender for each patient.

3.2. Immunohistochemical Methods

Immunohistochemistry was performed as described elsewhere (27): briefly, slides were deparaffinized and endogenous peroxidase was blocked by incubating them with 4% H_2O_2. Antigen retrieval was performed by microwave heating in citrate buffer. A panel of mouse monoclonal antibodies for mesenchymal stem cells was selected: CD34 1:50 (clone: QBEnd-10) (DAKO Norden, Denmark); CD44 1:200 (clone: 156-3C11); CD56 1:100 (clone: NCAM-1Ab2) (Thermo Scientific, USA); CD133 1:80 (clone: PAB1909); NGF-Receptor (p75 or CD271) 1:50 (clone: NGFR5) (Thermo Scientific, USA). Additionally, the following antibodies were used: Cytocheratin 1:50 (clone: AE1/AE3), CD45 1:50 (clone: 2B11+PD7/26) (DAKO Norden, Denmark), and Smooth Muscle Actin 1:100 (clone: 1A4) (DAKO Norden, Denmark) to rule out epithelial and myoepithelial cells; Ki67 1:200 (clone: MIB-1) (DAKO Norden, Denmark) to determine actively cycling cells.

Primary antibodies were incubated overnight at room temperature in BSA. Negative controls were incubated with an irrelevant primary antibody. Positive controls were run using human specimens carrying the antigens investigated. Monoclonal antibody binding was visualized using the Envision+ System HRP (Dakocytomation) with DAB (DAKO) as substrate chromogen. Sections were counterstained with hematoxylin. Each slide was examined under a multi-head light microscope and scored by two pathologists. Crisp staining above the negative control was scored as positive. A Masson Trichrome histochemical stain (BioOptica, Milan, Italy) was used to identify dense and loose collagen components.

3.3 .Cell counting

Immunohistochemical positivity was expressed as the percentage of positive fibroblast-like cells within 10 HPF (40x) fields randomly selected in areas of loose collagenous connective tissue. Positive and negative cells were counted separately . Positive perivascular cells were excluded from the count (see discussion).

4. RESULTS

Dental follicle tissue samples were taken from normal third molars extracted from 27 patients. At microscopic examination, follicles appeared as mesenchymal tissue with remnants of reduced enamel epithelium in the fibrous connective tissue layer. In some areas of loose collagenous connective tissue with scattered small blood vessels, immature fibroblasts were identified (Figure 1a).Immunohistochemical staining could most clearly be assessed in the immature connective tissue areas previously determined. Staining for at least one of CD44, CD56 and CD271 markers was positive in seven cases: the selected areas of immature connective tissue were highly representative, and showed immunohistochemical

Table 1. Clinical and laboratory data of 7 patients with at least 1 of the stromal phenotype markers positive

Gender, n (%)		Age, Years		Tooth Site n (%)				Marker positive cases, n, %								
M	F	Mean	range	18	28	38	48	CKAE1/AE3	SMA	Ki-67	CD34	CD44	CD56	CD271	CD45	CD133
1 (14.3)	6 (85.7)	19.01	16-25	1 (14.3)	1 (14.3)	2 (28.6)	3 (42.9)	0 (0)	0 (0)	0 (0)	0 (0)	6 (85.7)	7 (100)	3 (42.9)	0 (0)	0 (0)

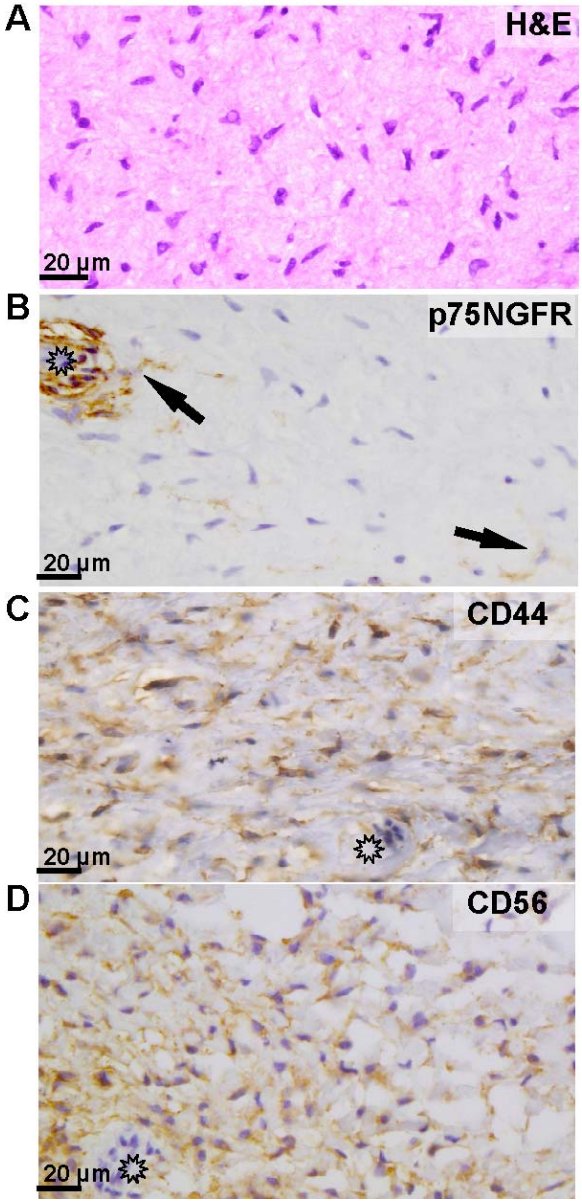

Figure 1. Representative examples for Hematoxylin and Eosin (A) and immunohistochemical staining for p75NGFR(B), CD44(C) and CD56 (D) on sections from dental follicle. The images show scattered p75NGFR positive cells (arrows) and CD44 and CD56 positivity in selected areas. Asterisks indicate blood vessels. Scale bar: 20 µm, magnification 40x

positivity (Fig 1b/c/d). In the cell count expressed as percentage of positive fibroblast-like cells, within 10 HPF (40x) fields randomly selected in areas of loose collagenous connective tissue, out of seven cases we observed a percentage of positivity for CD44 of 20.3%, for CD56 of 9.0%, and for CD271 of 0.7%. Table 1 shows clinical and laboratory data of the above mentioned 7 patients. Table 2 gives the percentage of cells positive for each of the 3 markers typical of stromal phenotype in these patients. In specimens from each individual patient, the tissue areas positively stained by each antibody widely overlapped, indicating co-expression on single immature mesenchimal cells. Table 2 reports details of patients and immunohistochemical results for the seven selected cases. Immunohistochemically-positive structures other than fibroblast-like cells (i.e. CD34 positive small vessels, CD45 positive leucocytes) were excluded from the assessment reported in Table 2. The results of cytokeratin and smooth muscle actin immunohistochemistry, which was performed to rule out epithelial and myoepithelial cells, were negative in all cases. Ki67 staining identified no active cycling cells; CD34, CD45, CD133 immunohistochemistry likewise showed no staining.

5. DISCUSSION

Studies performed with flow cytometry by Yalvac *et al.*, 2009, of the markers expressed by stem cells, found positivity for CD73, CD90, CD105 and CD166, and negativity for CD34, CD45, and CD133. A recent review on stem cells in dental tissues reported that dental follicle progenitor cells expressed Notch-1, CD13, CD44, CD73, CD105 and stro-1 antigens, but showed no expression of CD34, CD45 or CD133 antigens, which are usually expressed by bone marrow stem cells. Other studies, using the Fluorescence Activated Cell Sorter (FACS) technique (22,33), reported positivity for stro0-1, CD9, CD10, CD13, CD29, CD31, CD44, CD49d, and CD59, and negativity for CD34, and CD45. This finding is confirmed by other studies (25), and the data are in agreement with our observation of negativity for CD34, CD45, and CD133. With regard to CD 56 and CD 271, to our knowledge we are the first to test for them with immunohistochemical methods. Table 3 lists the dental stem cells markers reported by different studies. We report the identification of immature fibroblast cells with phenotypic features of stromal stem cells in the dental follicle. In our tissue samples, the markers were not uniformly expressed, but found in subsets of cells, in selected areas of morphologically immature, loose connective tissue, indicating that the DFC population is heterogeneous. In all cases, in the same selected areas of stem

cell marker positivity, the proliferation marker Ki67 was negative, suggesting that cells were not actively cycling. In the areas positively stained for CD44, CD56 and CD271, stem cells might account for a small fraction of the population of non-proliferating positively-stained cells. The immunohistochemical positivity was expressed as the percentage of positive fibroblast-like cells within 10 HPF (40x) fields randomly selected in areas of loose

Table 2. Percentage of cells positive for each of 3 markers in 7 patients

CASE n°	CD44 %	CD56%	CD271%
1	0.2	4.2	0
2	3.0	37.3	0
3	63.4	2.6	0.1
4	64.3	2.3	1.8
5	2.3	0.3	0.3
6	2.9	1.1	0
7	0	16.0	0
mean	20.3	9.0	0.7

Table 3. Dental stem cell markers identified in different studies

Marker	Reference numbers										
	13	14	26	28	22	23	24	34	25	35	Present study
Notch-1	IHC (+)	(RT) PCR (+)									
Nestin	IHC (+)	(RT) PCR (+)					(RT) PCR (+)				
Stro-1			IMF (+)	FACS (+)							
Vimentin		(RT) PCR (+)					(RT) PCR (+)				
Oct-4							(RT) PCR (+)				
ADAM28								PCR (+)			
p63										IHC (+)	
Ck AE											IHC (-)
SMA											IHC (-)
Ki-67											IHC (-)
ALP									FACS (+)		
HLA-DR									FACS (-)		
SH3									FACS (+)		
CD9				FACS (+)							
CD10				FACS (+)							
CD13				FACS (+)					FACS (+)		
CD14					FACS (-)						
CD15					FACS (-)						
CD29				FACS (+)	FACS (+)						
CD31				FACS (-)							
CD34					FACS (-)	FC (-)			FACS (-)		IHC (-)
CD44				FACS (+)					FACS (+)		IHC (+)
CD45				FACS (-)	FACS (-)	FC (-)			FACS (-)		IHC (-)
CD49d				FACS (+)							
CD56											IHC (+)
CD59				FACS (+)							
CD73						FC (+)	(RT) PCR (+)				
CD90				FACS (+)	FACS (+)	FC (+)			FACS (+)		
CD105				FACS (+)		FC (+)	(RT) PCR (+)		FACS (+)		
CD106				FACS (+)							
CD133						FC (-)					IHC (-)
CD146					FACS (+)						
CD166				FACS (+)	FACS (+)	FC (+)	(RT) PCR (+)				
CD271											IHC (+)

(RT)PCR= (Reverse transcription) Polymerase Chain Reaction , IHC= Immunohistochemistry, IMF= Immunofluorescence, FACS= Fluorescence Activated Cell Sorter, FC = Flow Cytometry, REF= reference, n=number, (+) positive, (-) negative.

collagenous connective tissue. In seven cases we observed a percentage of positivity for CD44 of 20.3%, for CD56 of 9.0%, and for CD271 of 0.7%. The association: negative for CD34, CD45, CD133, and positive for CD44 and CD56 is consistent with other reports (22,23,25,28), chiefly identified through *in vitro* methods. This association suggests these cells are mesenchymal stem cells, a hypothesis supported by the negativity of Ki-67 (proliferative index). The dental follicle comprises an ectomesenchymal tissue composed of various cell populations derived from the cranial neural crest. This tissue contains progenitor cells that display multidifferentiation potential and form the periodontium, i.e., cementum, periodontal ligament fibroblasts, and alveolar bone (29). Precursor cells have been isolated from human dental follicles of impacted third molars. As with other dental stem cells, after release from the tissue, only a small number of single dental follicle cells are attached to the plastic surface, and form few adherent clonogenic colonies. In *in vitro* characterization, cells within dental follicles express markers suggesting the presence of undifferentiated cells. DFPCs show typical fibroblast-like morphology; the markers expressed by these cells are usually investigated by flow cytometry. Immunohistochemical analysis with markers usually expressed by stem-cell cultures is an alternative way of investigating the presence of stem cells, and allows *in situ* analysis (30). A recent study on stem cells in dental tissues reported that dental follicle progenitor cells express Notch-1, CD13, CD44, CD73, CD105 and stro-1 antigens, but found no expression of CD34, CD45 or CD133 antigens, which are usually expressed by bone-marrow stem cells (19). This finding is confirmed by other reports (31-35) and is in agreement with our own findings: CD34, CD45, CD133 immunohistochemistry was negative. Nevertheless, characterization of DFCs showed that they express the markers for the mesenchymal stem cells CD44, CD56 and CD271. Stem cell marker positivity was found in patients irrespective of their age, sex and site of tooth extraction. CD44 has been reported to be expressed in DFPCs, while CD56 and CD271 have been found to characterize mesenchymal stem cells from other tissues. Pericytes or

fibroblast-like cells around small vessels have been claimed to have a stem-cell phenotype and have multilineage repopulating potential *in vivo* (34). We observed CD44+, CD271+ and CD56+ fibroblasts in this location, and thus we cannot rule out that some of the immature fibroblasts we detected may correspond to pericyte-like cells described in tissues other than the dental follicle. Stem cells have been reported to be a minute minority of terminally differentiated cells in epithelial and hematopoietic tissue. Very little is known for their representation in vascular or other mesenchymal tissues. We assume that not all immature dental follicle fibroblasts are stem cells, at least as assessed in an *in vitro* colony growth assay. Furthermore, not many tools are available to discriminate growth-arrested from senescent cells *in situ*, and thus, despite the suggestive phenotype, we cannot exclude that the majority of the cells we identified are not engaged in active stemness at the moment of the biopsy. Our results suggest the presence of immature fibroblast cells with phenotypic features of stromal stem cells in the dental follicle. By means of immunohistochemistry we identified some areas positively stained for CD44, CD56 and CD271, and stem cells might account for a small fraction of the population of non-proliferating positively-stained cells. However, further studies on a larger scale will be required to elucidate the molecular mechanisms involved in the maintenance and differentiation of these stem cell.

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