MORPHOLOGICAL AND CYTOFLUORIMETRIC ANALYSIS OF ADULT MESENCHYMAL STEM CELLS EXPANDED *EX VIVO* FROM PERIODONTAL LIGAMENT

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Many adult tissues contain a population of stem cells that have the ability of regeneration after trauma, disease or aging. Recently, there has been great interest in mesenchymal stem cells and their roles in maintaining physiological structure tissues and their studies have been considered very important and intriguing after having shown that this cell population can be expanded *ex vivo* to regenerate tissues not only of the mesenchymal lineage, such as intervertebral disc cartilage, bone, tooth-associated tissue, cardiomyocytes, but also to differentiate into cells derived from other embryonic layers, including neurons. Currently, different efforts have been focused on the identification of odontogenic progenitors from oral tissues. In this study we isolated and characterized a population of homogeneous human mesenchymal stem cells proliferating in culture with an attached well-spread morphology derived from periodontal ligament, tissue of ectomesenchymal origin, with the ability to form a specialized joint between alveolar bone and tooth. The adherent cells were harvested and expanded *ex vivo* under specific conditions and analysed by FACScan flow cytometer and morphological analysis was carried out by light, scanning and transmission electron microscopy. Our results displayed highly evident cells with a fibroblast like morphology and a secretory apparatus, probably indicating, that the enhanced function of the secretory apparatus of the mesenchymal stem cells may be associated with the secretion of molecules that are required to survive and proliferate. Moreover, the presence in periodontal ligament of CD90, CD29, CD44, CD166, CD 105, CD13 positive cells, antigens that are also identified as stromal precursors of the bone marrow, indicate that the periodontal ligament may turn out to be a new efficient source of the cells with intrinsic capacity to self-renewal, high ability to proliferate and differentiate, that can be utilized for a new approach to regenerative medicine and tissue engineering.

Haemopoiesis is sustained by two main cellular components, the hematopoietic cells and the mesenchymal progenitor cells (1-2). Mesenchymal progenitor cells (MPCs), precursors of mesenchymal tissues, are multipotent and represent the precursors for marrow stroma, bone, cartilage, muscles and connective tissue (3). To date, different authors have focused their attention on the precise identification of the biological properties of MSCs and specifically concerning the existence in the adult organism of their biological niche that serve as stem cell reservoirs (4). A complete characterization of the cellular, biochemical, and molecular interactions of MSCs within their niche is needed in order to understand how these cells can be optimally regulated *in vitro* and used for therapeutic approaches and gene therapy.

Despite the fact that bone marrow is considered a

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well-accepted source of MSCs, they have been isolated from other tissue sources (5-7), suggesting that MSC niche may not be restricted to bone marrow, but mesenchymal stem cells are diversely distributed *in vivo* showing a significant regenerative potential, treatment and cure of a variety of diseases of different tissues of the body (7).

In fact, it has been demonstrated that mesenchymal stem cells expanded *ex vivo* were able not only to regenerate tissues of the mesenchymal lineage, such as intervetebral disc cartilage (8), bone (9), and cardiomyocytes (10), but also to differentiate into cells derived from other embryonic layers, including neurons (11). Several approaches have been examined and viral transduction has been used, utilising an adenovirus vector containing dominant-negative mutant collagen type I to repair the bone in individuals with the bone disorder osteogenesis imperfecta (10), thus making it a popular option in gene therapy.

MSCs have the potential to produce different types of oral tissue and can be utilized clinically to repair or regenerate oral damage (12). In fact, previous experiments have shown that dental pulp tissue containing postnatal stem cells which are able to differentiate to osteoblasts/odontoblasts, adipocytes, and neuronal-like cells and in the periodontal ligament there is present a novel population of multipotent stem cells STRO1/CD146 positive with ability to develop into cementoblast-like cells and with the capacity to form collagen fibres, similar to Sharpey's fibres, connecting to the cementum like tissue.

The periodontal ligament is a soft, specialized connective tissue included between the cementum and the inner wall of the alveolar bone, it not only has an important role in supporting teeth and as sensory receptors, but also contributes to tooth nutrition, homeostasis, and the repair of damaged tissue is supported by stem cells migrating from the vascular region, as well as local cells (13-14). Since periodontal diseases are infectious diseases and are the main cause of tooth loss, the reconstruction of periodontium is a major goal of periodontal therapy. We have developed a novel approach in isolating purified populations of MSCs and we found that the study of their features by immunological and morphological analysis, carried out at light microscopy and at ultrastructural levels appear very interesting and highly useful for future research in tissue engineering.

MATERIALS AND METHODS

Cell culture

Samples of oral tissue were obtained from extractions after informed consent from patients. Extracted teeth were rinsed four times in phosphate – buffered saline (PBS) containing penicillin and streptomycin and subsequently the periodontal ligament was removed and cut into small pieces and incubated in 5% CO_2 atmosphere. Human mesenchymal stem cells were obtained and cultured according to the manufacture's directions in MSCM medium provided by the company (Cambrex Company, Walkersville MD 21793-0127).

After 20 days of cultures, numerous cells forming colonies (CFU-F) migrated from the explants. At day 7 adherent cells, which were 80-90% confluent by phase contrast microscopy, were isolated using 0.1% trypsin solution and plated in tissue culture polystyrene flasks at $5x10^3$ cells/cm^2. Primary cultures of PLD mainly consisted of colonies of bipolar fibroblastoid cells which, after subcultivation, proliferate with a population doubling time of 48h reaching a confluent growth-arrested condition.

Cells in the developing adherent layer were used for the experiments described after removal with 0.1% trypsin solution and cell growth was evaluated by trypan blue exclusion test.

Transmission electron microscopy

Cells were grown in the cellagen discs CD24 (ICN Biomedicals, Aurora OH), fixed with 2.5% glutaraldehyde in cacodylate buffer for one hours and post-fixed with 1% OsO_4 in the same buffer for one hours at 4°C. Samples were stained over-night with saturated aqueous uranyl acetate solution, dehydrated and embedded in Spurr's medium (Polyscience, Warrington, PA, USA)(15).

Thin sections were counterstained with lead citrate. Ultrastructural observations were carried out using a Jeol JEM 1010 electron microscope operating at 60 kV.

Scanning electron microscopy

The cells were washed in 0.1M phosphate buffer with 0.15M of sucrose to ensure the osmolarity remained at about 360 mOsm, prefixed for 20 h at 4° C in a 5 ml of glutaraldehyde at 2% in 0.05M phosphate buffer (pH 7.4) and after washed again with buffer solution. Following prefixation the specimens were treated with OTOTO method of post fixation as MALIK-WILSON (16) involving repeated exposure to osmium tetroxide and

thiocarbohydrazide. All specimens were than dehydrated using graded alcohol series and Critical Point Dried (CPD)from liquid CO_2 in an Emitech K 850 (Emitech Ltd. Ashford, Kent,UK). All samples were mounted onto aluminium specimens holders using carbon adhesive discs, and very lightly coated with gold in an Emitech K 550 (Emitech Ltd. Ashford, Kent,UK) sputter coater before imaging by means of a SEM (LEO 435 Vp Cambridge, UK)(16).

Flow cytometry analysis

Mesenchymal stem cells were washed in PBS and subsequently suspended in PBS with saturating concentrations (1:100 diluition) of the following conjugated mouse- anti human antibodies fluorescein isothiocyanate (FITC) conjugated: HLADr, HLAB27, CD2, CD3,CD7, CD34,CD14, CD45, CD117, CD44; CD29, STRO1, and CD105, CD33, CD90, CD166 phycoerythrin (PE) labelled, for 30 minutes at 4°C. The cell suspension were washed twice with PBS and analysed on a FACStar plus flow cytometer (Becton–Dickinson, Mountain View, CA, USA). All antibodies were from Becton Dickinson (17).

Experiments were repeated at least three times with at least for different donors with similar results.

RESULTS

It is interesting to point out that in this work we have employed a different method to isolate MSCs, developing a novel approach to isolate purified populations of adherent cells derived from the periodontal ligament displaying fibroblastoid features. In fact, we have observed that using a specific medium, a population of MSCs migrates from explants after 20 days of culture; all showing the same morphological and phenotypical features. All experiments were carried out with undifferentiated human mesenchymal stem cells guaranteed between 2 and 4 passages. Cells were grown at 200 cells/ml, detached from the well at 24, 48, 72 hours and cell growth and viability was determined by the trypan blue exclusion test (Fig. 1).

Under light microscopy, primary cultures of MSCs consisting of colonies of adherent cells show a morphologically homogeneous fibroblast- like appearance with a stellate shape and long cytoplasmic processes (Fig. 2, section A). Usually, the cells were adhering to each other and subsequently forming colonies (Fig. 2, section B). The nuclei are round or oval-shaped and the secretory apparatus is very well represented. Transmission electron microscopy studies of MSCSs grown on cellagen membrane show the characteristic adherent fibroblast–like cells. The elliptical nucleus contains the nuclear chromatin finely dispersed with one or two nucleoli. The cytoplasm is rich with mithocondria, rough endoplasmic reticulum and Golgi apparatus. Numerous roundish structures of different size and density were identified as lamellar bodies, they are associated with the Golgi apparatus and contain homogeneous and strongly electron-dense granules (Fig 2, section C and D).

Scanning electron microscopy confirmed the characteristic fibroblastoid-mesenchymal stem cells with many cytoplasmic processes (Fig. 3, section A and B). At higher magnification, the cytoplasmic elongation show several vesicles of different size and very long filopodia that take contact with neighbour cells (Fig. 3, section C).

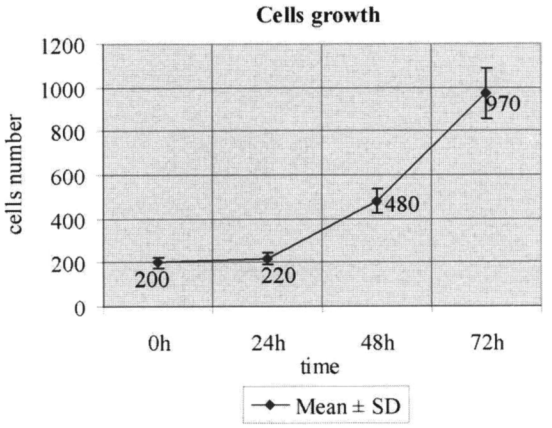

Fig. 1. *Analysis of cells proliferation during II passage of subculture at different time. It is possible note a logarithmic growth of MSCs. Counted cells show more than 98% viability (y axis, cell growth/ml, x axis, time of culture. Value obtained represent the average of three separate experiments ± SD.*

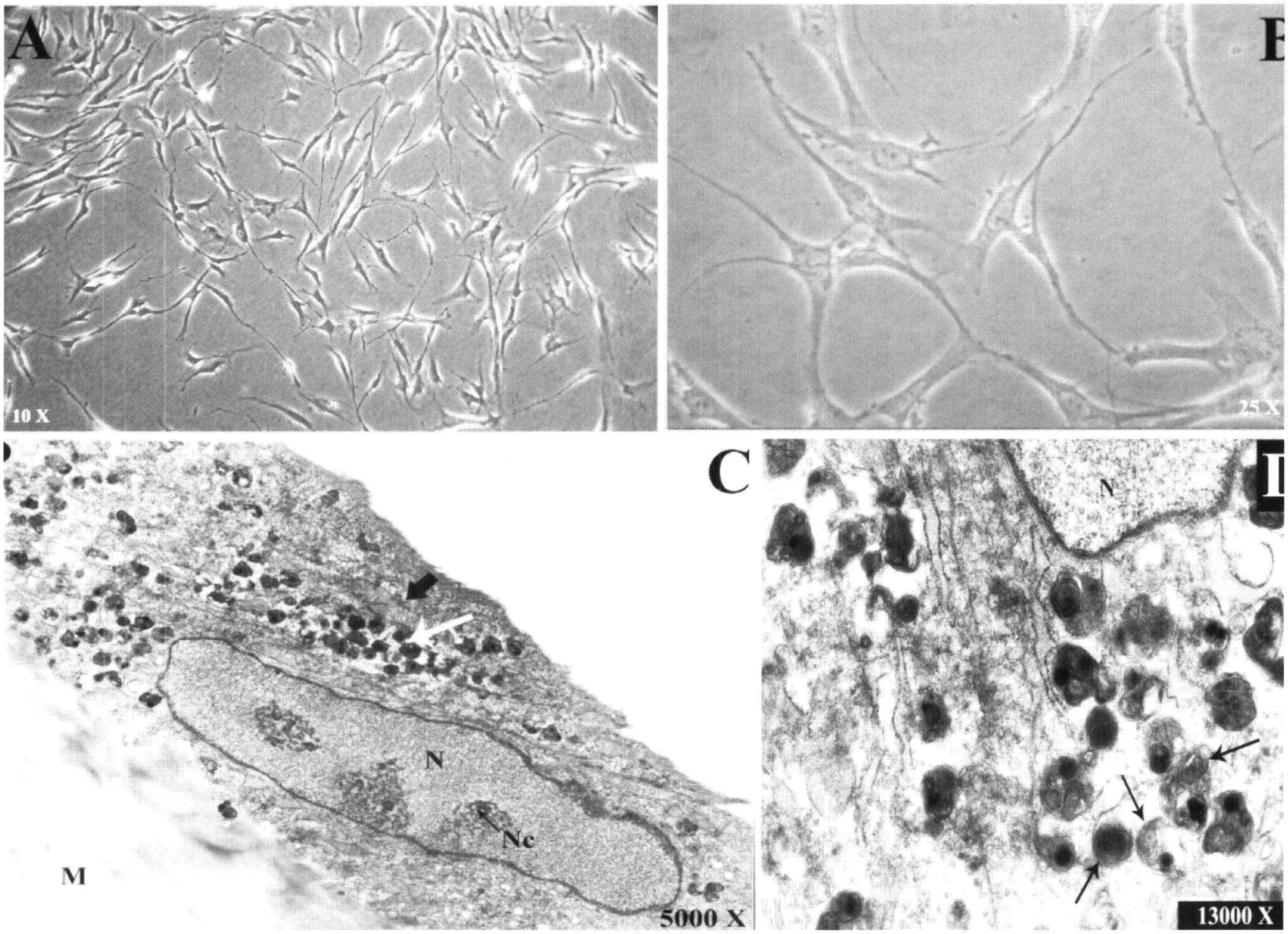

Fig. 2. *Section A: Mesenchymal stem cells, observed to inverted microscopy, display a fibroblastic morphology. Section B: MSCs exhibited the largest size and most well-developed endoplasmic reticulum and vacuolar apparatus. Section C: Transmission electron microscopy analysis of expanded ex vivo mesenchymal stem cells. Cells were grown on cellagen membrane (M). It is possible to visualize the specific features of adherent cells. The nucleus (N) contains the finely dispersed chromatin, two nucleoli (Nc), and numerous vesicles(white arrow). Section D: higher magnification of roundish structure present in the cytoplasm containing electron dense granules (arrows).*

Moreover, newly synthesized and secreted vesicles are present, probably the enhanced functioning of the secretory apparatus of the mesenchymal stem cells may be associated with the secretion of molecules required to survive and proliferate, probably involved in the paracrine and autocrine mechanisms (Fig. 3, section C and D) (18). The immunophenotype of these cells, as studied by flow cytometric analysis, disclosed the homogeneous expression of the mesenchymal-related antigens CD90, CD29, CD166, CD105, CD 44 (Fig. 4), did not express HLADr, HLAB27, CD15 , CD33,CD34, CD45, CD14,CD2,CD7, CD3, CD117, while CD13 was weakly positive (Tab. I).

A representative flow cytometry plot of mesenchymal stem cells visualized according to light scatter reflect cytoplasmic granularity and large cell size. (Fig. 4, section A).

The immunephenotype and morphological properties showed that MSCs cell expanded ex vivo from periodontal ligament were similar to bone marrow mesenchymal cells and human umbilical cord perivascular cells (7, 19-20).

DISCUSSION

It has become clear that adult mammalian bone marrow contains two populations of adult stem cells; one is responsible for maintaining a lifetime production of blood cells and the other, less well understood, is a resident population of stem cells known as bone marrow stromal cells or

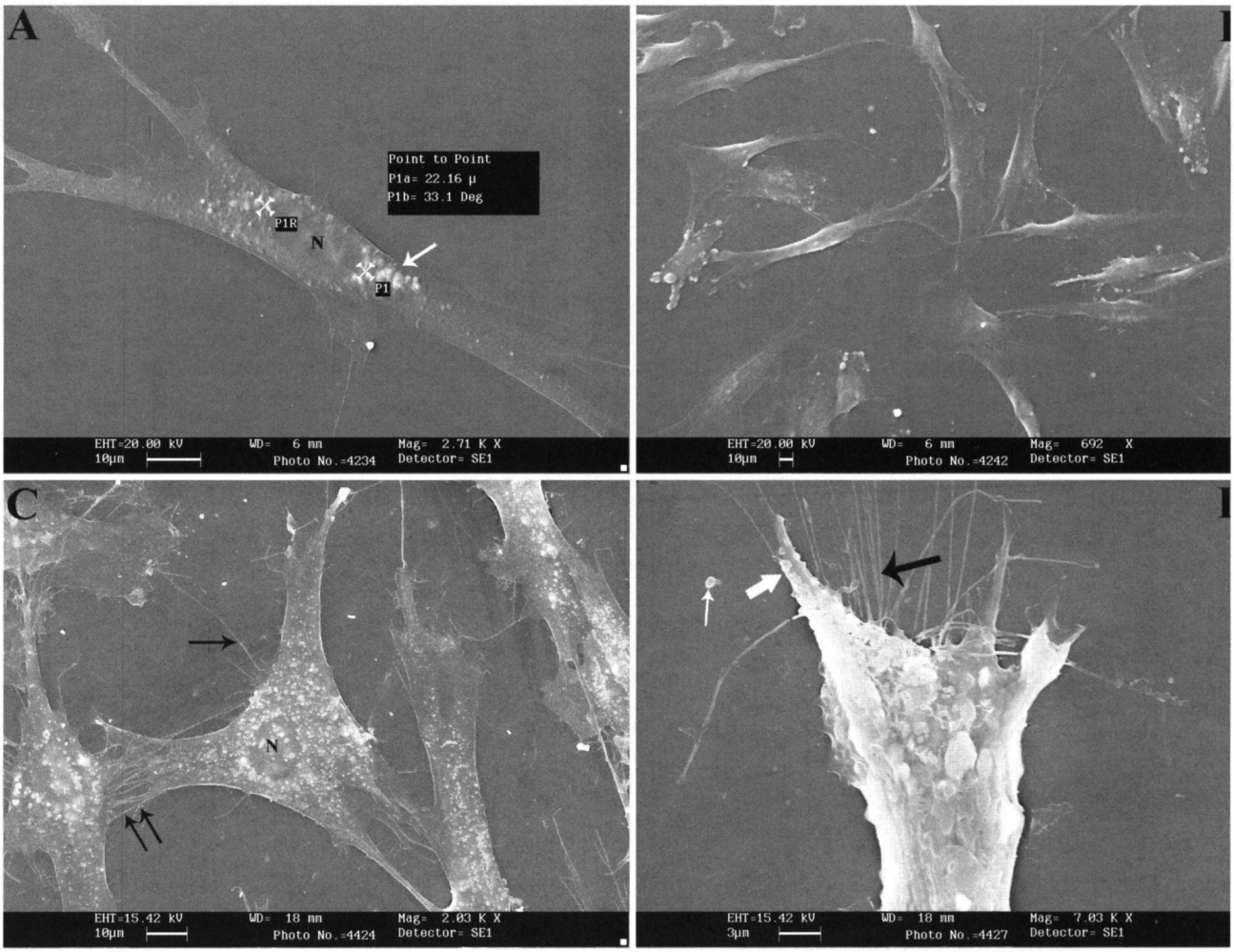

Fig. 3. *Section A: Scanning electron microscopy picture of mesenchymal stem cells of periodontal ligament with characteristic fibroblastoid morphology. The nucleus is oval-shaped having a size of about 22 mm, and presents an evident vacuolar system (white arrow). Section B and C show MSCs attached to each other, a well marked vesicular secretory pathway (asterisk), cytoplasmic processes and filopodia (arrow) Section D: higher magnification of cytoplasmic process showing numerous filopodia and cytoplasmic membrane swelling due to presence of numerous vesicles (white arrow). The thin white arrow illustrates a new secreted vesicles.*

mesenchymal cells (MSCs) (21). Due to their multipotentiality and capacity for self-renewal adult MSCs represent a promise for biological and therapeutic applications. In fact, stem cells have been shown to possess great potential for treatment and cure of various diseases and for their ability to differentiate into functional cell types that constitute different tissue. Numerous efforts, focused on identifying factors that regulate and control MSCs fate decisions, are crucial in promoting a greater understanding of the molecular, biological and physiological characteristics of this potentially useful stem cell type. Moreover, different authors have also shown that human umbilical cord perivascular cells represent a population of normal, rapidly expandable cells with intrinsic capacity of self-renewal and can furthermore generate multiple cells able to differentiate in different tissue (7,19-20). Recently, comparing the gene expression profiles by microarrays analysis of human dental pulp cells and bone marrow stromal cells, it has been demonstrated that they possess similar levels of gene expression in more than 4000 known human genes (22). Moreover, dental pulp is a viable source of easily attainable cells with a possible potential for cell transplantation therapies, indicating the presence of a cell population

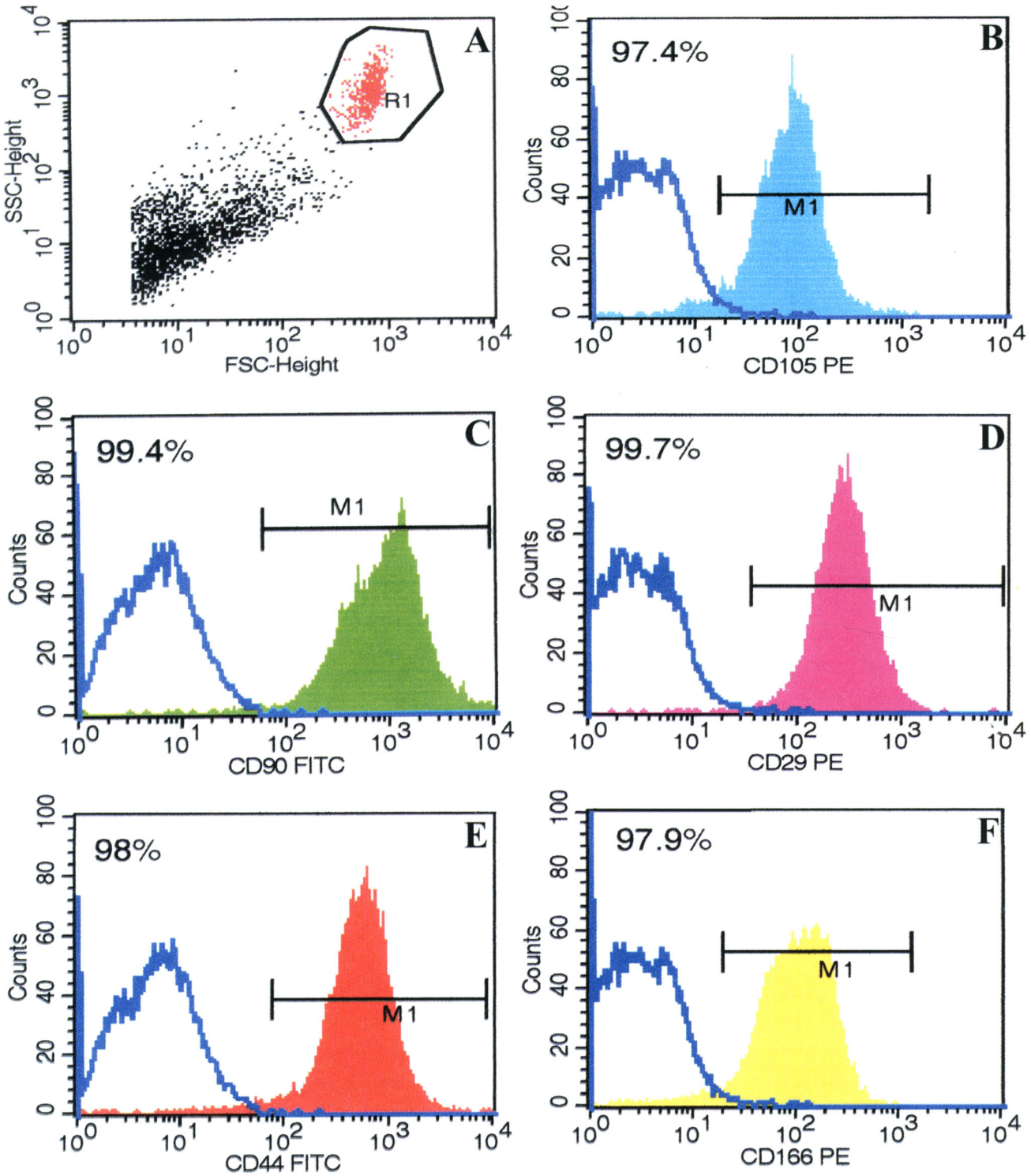

Fig. 4 . *Flow cytometry analysis of the mesenchymal-related antigens.*
Section A shows a representative flow cytometry plot of mesenchymal stem cells visualized according to light scatter characteristic reflect, cytoplasmic granularity and high cell size (R1). Sections B, C, D, E and F show the positive expression of MSCs to mesenchymal-related antigens: CD105 (97.4%); CD90 (99.4%); CD29 (99.7%) ; CD44 (98%); CD166 (97.9%).

Markers	HLADr	HLAB27	CD2	CD7	CD13	CD14	CD33	CD34	STRO1
Expression	--	--	--	--	15%	--	--	--	--

Table I. *Flow cytometry results of MSCs cells labelled for negative mesenchymal- markers (HLADr; HLAB27; CD2; CD7; CD14; CD33; CD34; STRO1) and weakly positive to CD13 (15%) antigen.*

in the dental pulp with neuronal differentiation capacity (23), and also having the ability to differentiate in mesenchymal tissue, after induction, offering a novel approach for regenerative medicine (24-26).

These results are in accordance with the existence in the adult organism of a biological niche of the mesenchymal stem cells that are not only restricted to bone marrow but are present in different tissue and under defined inductive conditions, are able to acquire characteristics of cells derived from embryonic mesoderm, as well as possessing ectodermal and neuronal properties (4, 27-30).

Recently, investigators have explained the multipotency of postnatal stem cells derived from periodontal ligament, and their possibility in periodontal tissue regeneration (13, 31). In fact, stem cells from periodontal ligament transplanted in rodents have the capacity to differentiate and have exhibited the ability to generate cementum/PDL-like structures that may assist in periodontal tissue repair, demonstrating the ability that these cells might have in dental tissue engineering (31). Mesenchymal stem cells from periodontal ligament are able to form calcified deposits in vitro as has been seen in other mesenchymal stem cells derived from bone marrow and dental pulp (12-13).

In this paper, apart from cytofluorimetric determination, we report the isolation and characterization, by morphological analysis, of homogeneous mesenchymal stem cells population expanded *ex vivo* from periodontal ligament. Our approach provides an alternative strategy to expand adult MSCs from oral tissue in a logarithmic and controllable bioprocess and also provides new and interesting possibilities to explore mesenchymal stem/progenitor machinery in the cell biology. In fact, using a morphological analysis we have for the first time, characterized mesenchymal stem cells of periodontal ligament offering the possibility to understand all their morphological features and a possible correlation with their function. More important, these cells are an ideal carrier for delivering genes into the tissues for gene therapy applications and tissue engineering. They are directly obtained from individual patients and can eliminate all complications associated with immune rejection of allogenic tissue and would not induce any immunoreactivity in the host upon local transplantation or systemic administration.

Further studies, to enhance their clinical usefulness, are being carried out in our laboratory from evaluating the maintenance and differentiation of this cell population and the signal transduction regulating their mechanism *in vivo* and *in vitro*.

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