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Archives of Stem Cell Research

Review Article

Human Periodontium-Derived Stem Cells May Give Rise to Carcinoma

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Submitted: 23 July 2015

Accepted: 10 August 2015

Published: 12 August 2015

Keywords

- Periodontium
- Stem cells
- Carcinoma
- Animal model

Abstract

The presence of stem cells in the periodontal ligament was supported by several findings where a opulation of mesenchymal stem cells from the periodontal ligament has been isolated and characterizedshowing the ability to express a variety of stromalcells markers. The in vivo regenerative capacity of human periodontium-derived stem cells (PdSCs) was investigated by using 10-week-old athymic nude rats that served as an animal model for cell transplantation. Two animals from the second group (sacrificed at week#6) and all the animals of the third group (N=4) (sacrificed at week#8) presented remarkable tissue enlargements exactly at the operation/test side where PdSCs had been previously transplanted. Histologically, these unusual tissueswere considered to be types of anaplastic squamous epithelial-cell carcinoma.The patients from whom the PdSCs had been extracted, the animal model used, and a possible oncogenic alteration of the PdSCs themselves might all be factors behind the tumors' initiation. Considering such tumors as cancer stem cells needs further investigations.

INTRODUCTION

The periodontal ligament (PDL) is a group of specialized connective tissue fibers that essentially attach a tooth to the alveolar bone within which it sits. These fibers help the tooth withstand the naturally substantial compressive forces which occur during chewing and remain embedded in the bone.

Using a methodology similar to that utilized to isolate Mesenchymal stem cells (MSCs) from deciduous and adult dental pulp, multipotent postnatal stem cells from human periodontal ligament, or PdSCs, have also been isolated and described [1-6]. Cultured cells were expanded from single cell suspensions derived from periodontal ligament tissue and the presence of stem cells was determined using antibodies such as STRO-1 and CD146. In recent works [7,8], PdSCs were isolated from human periodontal tissues and cultured as spheres in serum-free medium. After 10 days the primary spheres were dissociated and the secondary spheres sub-cultured for another 1–2 weeks. It was shown that PdSCs were capable of differentiating into osteogenic precursor cells, which produced extracellular matrix containing Osteopontin.

However, the regenerative ability of such cells and/or the side effects that may arise through using PdSCs *in vivo* is still uncovered.

Possible in vivo actions of PdSCs

The *in vivo* regenerative capacity of human adult PdSCs was investigated by using 10-week-old athymic nude rats (Charles River Laboratories, Research Models and Services, Sulzfeld, Germany). For surgical procedures, rats were fully anesthetized, and an artificial bone defect (about 2.5×2.5×2mm) was prepared on the right side of the mandible (as a test side) as well as on the left side (as a control side) at the level of the distal root of the first molars. Four days before transplantation, human adult PdSCs were dissociated and re-suspended in osteogenic differentiation medium as previously described [9]. Subsequently, osteogenic pre-differentiated PdSCs (1×10^5 cells/ml) were plated on OptiMaix® collagen sponges (Matricel, Herzogenrath, Germany),

which were previously fit to the shape of the bone defect, and were cultured for additional four days in osteogenic differentiation media. Collagen sponges without PdSCs served as controls in the mouth-split model. The collagen sponge with PdSCs was then transferred to the bone defect created on the test side, whereas the collagen lattice without cells was transferred to the defect created on the control side (Figure 1).

In total, 17 rats were used, whereby 16 rats were considered as test group, and 1 rat served as a control (no implantation of PdSCs). The regenerative capacity of PdSCs was investigated for 2, 6 and 8 weeks.

Immunohistochemistry

The tissues chosen for immuno-histochemical examination were fixed in 3.7% paraformaldehyde for 15 min, washed with phosphate buffered saline (PBS), incubated with 5% goat serum, 0.1% Triton® X-100to permeabilize eukaryotic cell membranes and then blocked and incubated with primary antibodies (mouse anti-human mitochondria, clone 113-1, Chemicon Inc.) at dilution about 1:50 for 1 h. The samples were subsequently incubated with goat secondary antibodies of anti-mouse CY.3 whole IgG (Jackson immunolabs Inc.) at dilution about 1:300 for 1 h. For enzymatic immunohistochemical staining, the Sytox® (Molecular Probes Inc.) 1:20000 was used according to the manufacturer's protocol.

For isolation of cell nuclei in order to count the number of chromosomes, cells were incubated after adding Colcemid for 4-6 hours and harvested afterwards. The cells were centrifuged and washed once with PBS. Pelleted cells were resuspended in KCl and incubated for 30 minutes at room temperature before pelleting them again. The cell pellet was resuspended carefully in the remaining KCl solution. MeOH/glacial acetic acid (3:1) was added drop by drop while mixing the cell suspension cautiously. Cell suspension was washed twice with MeOH/glacial acetic acid (3:1) and was resuspended in MeOH/glacial acetic acid (3:1). The cell suspension was dropped from a height of about 30 cm on top of a glass slide prepared with an H_2O sprinkled surface. The glass slide was dried and stored at 4°C. Chromosomes were stained with SytoxGreen and were counted by using a confocal

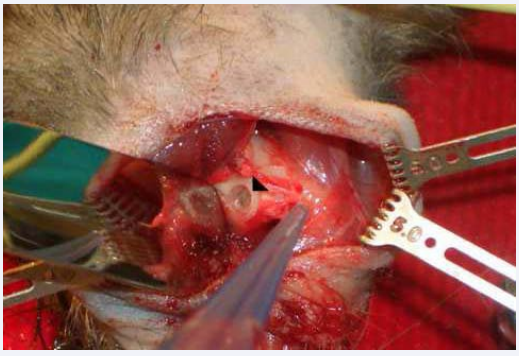

Figure 1 Performance of the artificial (periodontal) bone defect reaching the distal roots of the first molar (black arrow).

Figure 2 Six rats presented tumors on the test sides at W6 and W8 after PdSCs' implantation (in this figure, only 2 rats are demonstrated).

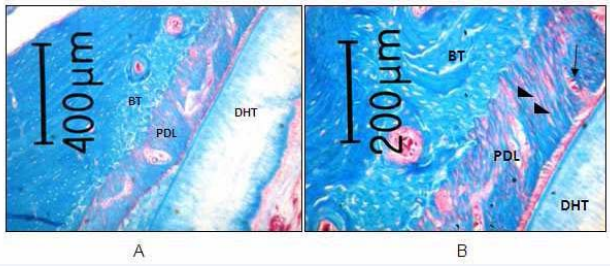

Figure 3 Histological section taken from the control rat (Nc) demonstrating normal periodontium in (A): (orig mag ×2.5) and (B): (orig mag ×5) (*). Collagen fibers (black triangles) and blood vessels (black arrow) can be shown within PDL (BT: Bone Tissue, DHT: Dental Hard Tissue, PDL: Periodontal Ligament).

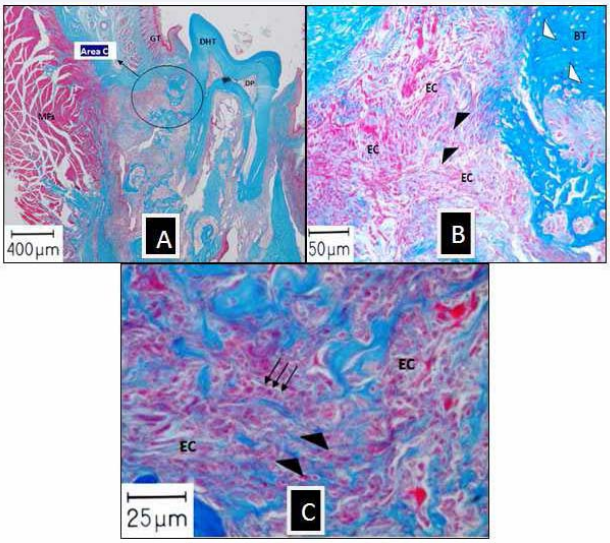

Figure 4 (A): Histological section taken from a test site which clinically showed tumor (*) (orig mag ×2.5), (B): Area "C" under higher magnification showing little bone tissue elements (BT), osteocyte-lacunae features (white triangles), area of dense infiltration with epithelial-like cells (EC) interrupted by fillets of collagen fibers (black triangles) (orig mag ×20), (C): Part of area "C" under higher magnification showing dense infiltration of undefined epithelial-like cells (arrows) and collagen fibers (triangles) (origmag ×40) (DHT: Dental Hard Tissue, DP: Dental Pulp, GT: Gingival Tissue, MFs: Muscular Fibers) and (Area "C": The area where collagen sponge with PdSCs was transplanted).

laser scanning microscope.

RESULTS

In general, all the rats showed normal and healthy activities before the operation. Five rats were excluded from the study because of post-operative death and contamination. On the other hand, all other rats (i.e. 11 rats) were successfully operated without any surgical complications before, during or after the operation.

At the time point of sacrificing the animals, the first group (N=3) (sacrificed at week#2) did not show any distinctive features, whereas two animals from the second group (sacrificed at week#6) and all the animals of the third group (N=4)(sacrificed at week#8) presented remarkable tissue enlargements exactly at the operation/test side (i.e. right side of the mandible). These tissue enlargements (later considered as tumors) were subcutaneous, a little bit hard when palpated, and, in some animals, distributed toward the neck area with different sizes (Figure 2).

All animals which presented those tumors suffered from weight's loss (about 40% of the original weight), and seemed to be fatigued. All tumors were lately dissected, weighed and isolated as separate specimens for histological examination.

Under light optical microscope the following results could be shown:

Sections taken from the control rat clearly demonstrated all histological features related to a normal periodontium; namely the periodontal ligament, the supporting bone and the dental hard tissue (here: dentin covered with cementum). Within the periodontal ligament, collagen fibers and blood vessels could be shown (Figure 3).

All the sections taken from the test sites of the third group as well as those taken from two sections of the second group (where tumors showed up) presented a tissue that contained remarkable invasion of undefined type of cells seemed primarily to be of epithelial-origin, very slight bone elements and some collagen fibers. This unusual tissue was considered to be a type of anaplastic squamous epithelial-cell carcinoma (Figure 4).

The biggest tumor (weight=6g), which belonged to a rat from the third group, was chosen to be referred to the immunohistochemical examination according to the method described in the previous section.

The molecular screening of this tumor showed a remarkable positioning of the human cells (i.e. the PdSCs) within the tumor tissue. The percentage of those cells was about 7.96±0.4% (Figure 5).

The chromosomal number of the cells involved in the tumor has been detected by means of a karyotype analysis (method mentioned above) and it has been shown that those PdSCs, which have been shown to form tumors, exhibited an aneuploidy karyotype with chromosome counts peaking at 70 chromosomes (Figure 6).

DISCUSSION

Little is still known about side effects which may reside within the periodontal ligament-derived stem cells' colony when considered for periodontal tissue regeneration [10].

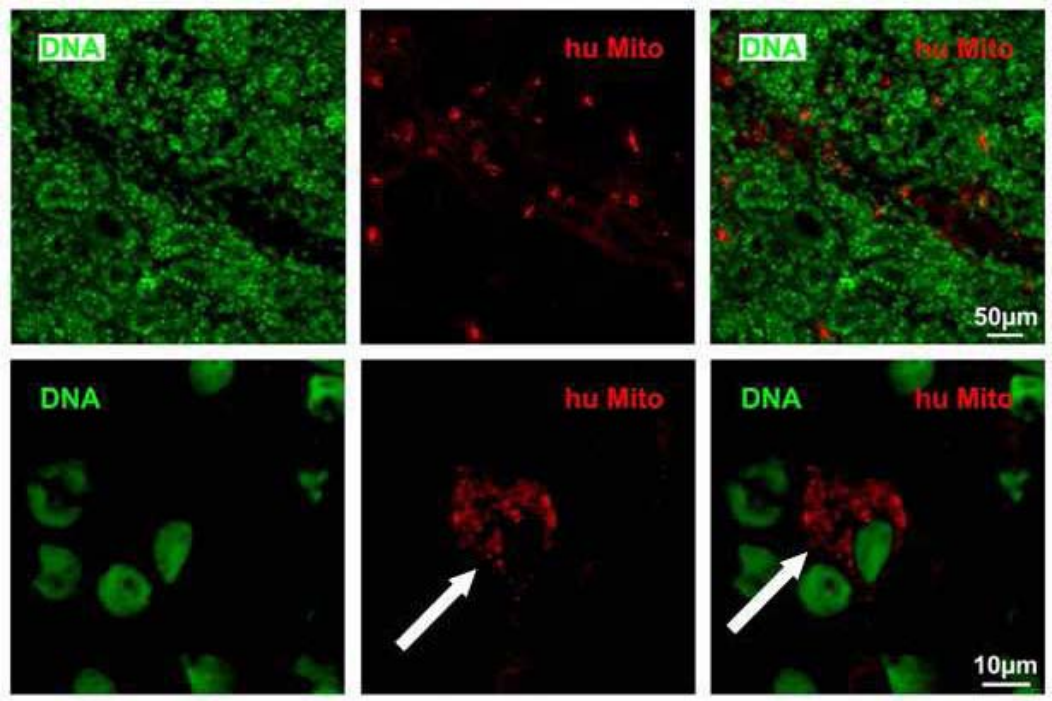

Figure 5 Immuno-histochemical staining of the tumor extracted from the rat#3.3 showing the presence of human mitochondria antigens (stained red) among the rat cells (stained green).

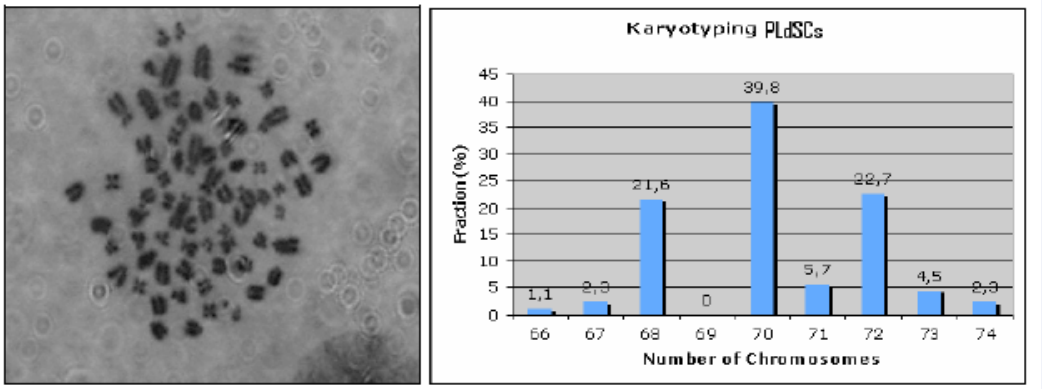

Figure 6 Karyotype analysis of PdSCs showing aneuploidy karyotype with chromosome counts peaking at 70 chromosomes.

In the current study, and because human periodontal stem cells were used, it was important to choose an animal model that had fewer ability to reject foreign transplantations. For this reason, immuno-deficient rats were the method of choice since they have no T-cells in their immune systems.

The current model of periodontal defect looks similar in design with the defect model described in a past study related to periodontal healing [11].

Six animals out of 11 presented remarkable tissue enlargements (later referred to as tumors) at 6 and 8 weeks after PdSCs implantation exactly at the right side of the mandible which was considered the "test" side. However, the initial visual and manual examination could not tell if those external tissue alterations were just inflammatory reactions or considerable as tumor initiation. The weight loss and fatigue that accompanied those distinctive features have led to the thinking that a local neoplastic alteration within the involved tissues has been occurred, since in late-stage cancer, it is believed that tumor- or stromal cell–derived molecules disturb the stringent control of appetite and weight control, leading to wasting, debility and often death [12].

Under light optical microscope, the histological sections taken from the test sides of the rats which presented the tissue enlargements showed a tissue with dense infiltration of epithelial-like cells interrupted by fillets of collagen fibers. Those features were considered to present a classical histological screen of carcinoma.

By definition, a carcinoma is any malignant cancer that arises from epithelial cells. Carcinomas invade surrounding tissues and organs and may metastasize, or spread, to lymph nodes and other sites.

Severely anaplastic tumors might be so undifferentiated that they do not have a distinct histological appearance. In the current study, the histological screening of the tumors initiated showed undifferentiated features so that the tumor type was considered as (undifferentiated type of carcinoma).

Malignant neoplasms that are composed of undifferentiated cells are said to be anaplastic. Lack of differentiation, or anaplasia, is considered a hallmark of malignancy.

In our study, the tumors detected were considered to be anaplastic type of carcinoma and might be malignant because they were less differentiated and - under optical light microscope - not clearly characterized.

We hypothesize that 3 major factors could be responsible for this malignant initiation; 1) the patients who played the role of PdSCs donors, 2) the rat model used and 3) the PdSCs themselves.

The PdSCs used in the current study were extracted from 10 adult patients (27-56 years old) who visited the Department of Periodontology at the University of Witten/Herdecke seeking a complete periodontal treatment, of which surgery was an important part. All the patients were suffering a severe type of chronic periodontitis which means that the PdSCs were extracted from severe inflamed tissues.

Inflammation can play a role in tumor suppression by stimulating an antitumor immune response, but more often it appears to stimulate tumor development. Interestingly, inflammation functions at all three stages of tumor development: initiation, progression and metastasis. Inflammation contributes to initiation by inducing the release of a variety of cytokines and chemokines that alert the vasculature to release inflammatory cells and factors into the tissue milieu, thereby causing oxidative damage, DNA mutations, and other changes in the microenvironment, making it more conducive to cell transformation, increased survival and proliferation.

The link between infection, chronic inflammation and cancer has long been recognized [13], a prime example being infection with Helicobacter pylori (*H. pylori*) and gastric cancer [14]. Chronic gastric inflammation, which develops as a consequence of H. pylori, leads over time to repetitive injury and repair resulting in hyper-proliferation, an increased rate of mitotic error, and progression to adenocarcinoma.

Houghton and colleagues' work [15] suggested an alternative to the inflammation theory. The research group focused on the idea that bone-marrow-derived cells move into areas of chronic injury or inflammation to effect repairs. A strain of mice (C57BL/6) and a relative of *H. pylori* (*H. felis*) had been used so

that, together, they formed a well-established model of gastric cancer in humans. It has been shown that the implanted bone marrow-derived stem cells migrated first into the stomach lining, presumably to repair the damage caused by the bacteria; by 20 weeks, the labeled cells were differentiating into cells with the characteristics of stomach epithelial cells. These differentiating cells showed a high rate of growth, and after 52 weeks, the mice were in the early stages of developing gastric cancer, and the tumors that subsequently formed stained positively for markers that indicated that the cells indeed came from the bone marrow.

At the same level, Dittmar et al. [16] discussed a model showing that bone marrow-derived stem cells might contribute to overall tumor development due to recruitment to tumor tissue.

Other study showed that in chronically inflamed skin, or in an immuno-deficient patient, malignant transformation of extra-cutaneous stem cells is more likely to occur [17].

All these data suggest that an inflamed environment could change the manner of stem cells and subsequently enhanced the (inflammation/infection- into-tumor) cascade. A genetic mutation might have played a role at this level.

The rat model used in the current study may be a hidden reason that explains the tumors' initiation. The notion that the immune system could protect the host from neoplastic disease was initially proposed by Ehrlich et al. [18] and formally introduced as the cancer immuno-surveillance hypothesis nearly 50 years later by Burnet et al. [19]. Tumor development in mice had been shown to be controlled by components of the immune system like interferon-γ (IFN-γ), Perforin, Natural Killers (NK) and Lymphocytes. Girardi and co-workers examined the relative contributions of different T-cell subsets in blocking primary tumor formation in mice lacking different types of T cells and showed that those mice have more tendencies to initiate tumors [20]. Moreover, it has been suggested that the fate of developing pre-cancer stem cells (pCSCs) is determined by the status of the host immune system and the environmental cues (the site of cell colonization or route of inoculation) [21]. In their study, the pCSCs appeared to be scrutinized by the mechanism of tumor immune surveillance because they had different fates in three animal models with different levels of immune surveillance; they developed into neither solid nor leukemic tumors in native immuno-competent mice. However, they developed into tumors or leukemia in the NK cell-sufficient but T and B cell-deficient mice with a variable latency of tumor initiation and tumor incidence in separate experiments [21].

Since the rats used in our study are T-cell deficient and show depleted cellpopulations in the thymus-dependent areas of peripheral lymphoidorgans, it could be stated that this immunodeficiency might be the reason why the tumors occurred.

The presence of PdSCs within the solid tumor may give a sign that those stem cells could be responsible for the initiation of the neoplasias detected in the rats. Whether those tumors can be considered as "stem cells-induced tumors" is not clear and needs further immunological examinations [10]. However, the presence of human mitochondria antigens in the solid tumor may lead to the supposal that the implanted human PdSCs might have played a role at this level.

According to the Cancer Stem Cell (CSC) theory, both adult stem cells and progenitor cells are proliferating cells, and therefore go through sufficient cell cycles to accumulate oncogenic mutations during their life. The adult stem cells are long-lived cells going through relatively few cell cycles, and may be the primary targets of accumulation of oncogenic mutations [22-25]. Adult stem cells and tissue-uncommitted stem cells might be more susceptible to developing into pre-cancer stem cells and CSCs than other progenitors [26], and the pCSC might be more plastic than CSCs.

Our results, showing that PdSCs exhibited aneuploidy karyotype with chromosome counts peaking at 70 chromosomes, and that the human cells (i.e. the PdSCs) reside within the tumor tissue, might also be of great importance.

According to all these data, and because we used human adult stem cells in this study which have been previously shown to be highly proliferative [27], it might be stated that the PdSCs and/or their progenitors have underwent critical alteration(s) which has (have) redirected their development into tumor initiating cells. We also hypothesize that the method used to expand PdSCs *in vitro* in our study might be a factor that altered the proliferation form of those cells when implanted in vital tissues.However, deep investigation of the tumors observed in the current results as well as analyzing their characteristics were not the definite priorities of this work. Further genetic analyses in this term are needed to uncover the real chromosomal instability presented in our model.

CONCLUSION

Remarkable notifications regarding the results obtained in our study were the induction of malignant tumors (squamous cell carcinoma) in 6 out of 11 animals. Considering the data presented in the literature, our study seems to be the first that demonstrates the initiation of malignant tumors when using human periodontal ligament-derived stem cells. The patients from whom the PdSCs had been extracted, the animal model used, and a possible oncogenic alteration of the PdSCs themselves might all be factors behind the tumors' initiation.

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Cite this article

Dannan A, Grimm WD, Becher S, Gassmann G, Arnold W, et al. (2015) Human Periodontium-Derived Stem Cells May Give Rise to Carcinoma. Arch Stem Cell Res 2(2): 1013.