ESTABLISHMENT OF STEM CELLS EXFOLIATED FROM DECIDUOUS TEETH PULP

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SUMMARY

The pulpo-dentinal complex has protective qualities safeguarding the dental pulp from progressive inflammations and traumatic lesions. This effect is achieved through the formation of a dentine-like structure made-up of cells capable of additionally differentiating. It is supposed that these are multipotent stem cells. The isolation and proving the existence of such cells is of crucial importance for regenerative medicine.

It is the aim of this project to prove the presence of the primary structures of mesenchymal stem cells from the pulp of temporary teeth and characterising as well as determining the impact of growth factors TGF-в2 and BMP-2 on the model system studied.

Immuno-fluorescence, analysis of the cell proliferation and a vitality test (MTT) were conducted. The impact of BMP-2 (Bone Morphogenic Protein-2) and TGF-в2 (Transforming Growth Factor-в2) on the isolated and cultivated colonies of stem cells was determined.

The project accomplished succeeded in isolating stem cell from the pulp of exfoliated temporary teeth. Such cells when in norm manifest no proliferation capacity. Stimulated with the help of growth factors, the cells come to manifest a clear potential for proliferation, though, and later become capable of multipotent differentiation.

Research in the last couple of years (14), including such conducted by ourselves, prove the presence of stem cells in the pulp of deciduous teeth to be physiologically shed. Hence the important task scientific research has been facing recently: to determine whether the multipotent cells can turn into pluripotent. The evidence collected so far is contradictory (1, 2). It seems that most multipotent cells of grown-up individuals have their limitations that make them capable of producing only a couple of types of cells. There are researchers who have found out that under certain conditions of cultivation multipotent cells can produce different types of cells (19).

Another important issue in these researches is the determination of the active products that are capable of stimulating the differentiation of the stem cells in an adult individual.

It is supposed that factors and signal molecules similar to those active in the physiological dentinogenesis, such as the TGFbs (transformational growth factos – beta) and the related with them proteoglicans IGF-I and II, members of the family of BMP, participate in the activation of the odontoblast during the process of reparative dentinogenesis. The supposition is that these factors and signal molecules separate within the dentine matrix and are released during the dentine dysmineralisation (15, 22).

Laboratory tests have been made until now proving in vitro that the cells from the dental pulp can differentiate into odontoblast under the impact of dexametason, ascorbic phosphate, inorganic phosphate and and BMP-4 (5, 6, 7). Cells from thepulp of wisdom-teeth are usually used in such tests (13).

Shi makes the discovery (14) that each pulp from a deciduous tooth has about twenty cells with much better qualities than the cells obtained from the pulp of a permanent tooth.

The stem cells from the functionally exfoliated deciduos teeth or SHED (Stem cells from human exfoliated deciduous teeth) represent postnatal stem cells and differ considerably in terms of qualities from the stem cells from grown-up individuals. They have the quality of growing much quicker and triple when cultivated. This shows that SHED probably are in a much less mature phase compared to the stem cells from adult individuals (14).

When differentiating factors are added to the medium an odonblast differentiation of the mesenchymal cells from the dental pulp of deciduous teeth is achieved (6, 7, 10, 12).

Goal:

It is the aim of this research to establish and characterise stem cells exfoliated from the pulp of deciduous teeth and to establish the impact growth factors TGF-в2 and BMP-2 have on the model system studied.

MATERIAL AND METHODS:

Immunofluorescence.

The cell cultures from the pulp of deciduous teeth were cultivated on petri dishes and after reaching sunconfluent condition were fixated by means of 4% formaldehyde. For immunofluorescence the following antibodies were used: goat polyclonal anti-Alkaline Phosphatase antibody (RnD Systems), mouse monoclonal anti-C-kit antibody (Santa Cruz Biotech. Inc.), anti-Nestin antibody (Santa Cruz Biotech. Inc.), Donkey anti-Mouse TRITC conjugated antibody (Santa Cruz Biotech. Inc.), Donkey anti-Goat antibody (Santa Cruz Biotech. Inc.), Alexa Fluor 488 phalloidin and Sybr green NAS (Invitrogen).

Analysis of the cell proliferation.

The cell proliferation was established by means of the method of measuring the inclusion of ^{3}H-timidin in the newly synthesised DNA of the proliferating cells. The cells were treated with a set of mitogenic factors for a period of 24 - 48h. During the last four hours of the treatment radioactively-marked timidin - 1μCi ^{3}H-timidin - was added to the cell cultures. By means of trichloroacetic acid the cells were fixated and cleansed from the radioactive timidin unabsorbed by the DNA and then lysined in 1N NaOH+1%SDS. The radioactivity of each sample was measured by means of scintillation meter Beckman. The degree of absorption of the radioactively marked timidin by the molecules of the proliferating cells' newly synthesised DNA was determined per mcg of protein as correlated with non-treated control cells.

Vitality test (MTT)

The cell cultures were grown up in 24 petri dishes. After reaching a subconfluent condition MTT was added with a final concentration of 0,5 mg/ml. After 3 hours a reduction of the MTT to a purple formasan was manifested. Registered was through ELISA at 570nm. The colorometric experiment with MTT - 3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide – determined the activity of the different dehydrogenous enzymes and is based on the fission of the tetrasole ring of the MTT, which happens only in the active mitochondria.

RESULTS AND DISCUSSION

In order that it is found out whether the medium can stimulate cell differentiation the activity of the alkaline phosphatase was studied. Alkaline Phosphatase (AP) hydrolizes different phosphatic esters and realises the transfer of phosphatic radicals onto another acceptor under alkaline pH. The activity of AP is highest in the liver, the bones (osteoblasts), the afterbirth and the red epithelium. The non-differentiated human embryonic cells evince high levels of AP activity. The activity of alkaline phosphatase drops with the differentiation of the cells. AP is described as a tetramer strongly connected by means of a hydrophobic part of its molecule to the membranous structures of the cell. This enzyme is widely used as a marker for cells capable of forming a mineralised structure such as the odontoblasts. The intensification of the activity of alkaline phosphatase is an important early marker for the stage of differentiation of the embryonic cells and is indicative of the function of both the osteoblasts and the odontoblasts. The registering of an increased reactivity towards alkaline phosphatase is indicative of there being non-differentiated pluriopotent stem cells.

The results obtained show the clear and intensive alkaline phosphatase in cells isolated from the pulp of a deciduous tooth (Fig. 1).

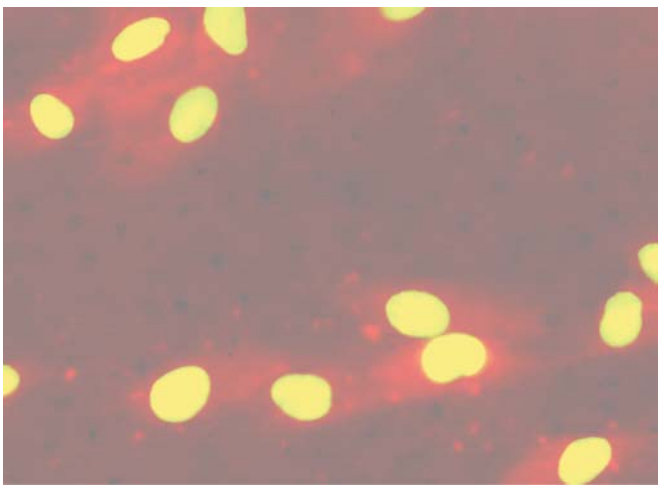

Fig. 1. Immunofluorescence of pulp mesenchymal cells for alkaline phosphatase (red). The nuclei of the cells are coloured in green.

The green pigmentation of the cells is indicative of the hightened activity of the alkaline phosphatase. This has to do with the functions which this enzyme performs within the process of cellular differentiation into osteoblast and odontoblast. The increased activity of the alkaline phosphatase shows that the cells isolated from the pulp of deciduous teeth have not only the capability to proliferate but also the capability to differentiate to cells that participate in the processes of mineralisation. This is a prove of the fact that the postnatal stem cells from the pulp of a deciduous tooth can be stimulated to differentiate to odontoblast and be used for the regeneration of the pulp affected with inflamation.

Another marker for the differentiation of the stem cells is the stem-cellular factor, known as C- kit (CD-117). It is a tirosine-kinase receptor that connects the SCF (Stem cell factor). It is represents a cytokinin responsible for the occurrence of spontaneous differentiation in the embrionic stem cells. It has two form – one connected with the cell surface and the other one dissoluble or free, being formed through the division of the form attached to the surface. SCF is a growth factor important for the survival of the cell, the proliferation and differentiation of the stem cells.

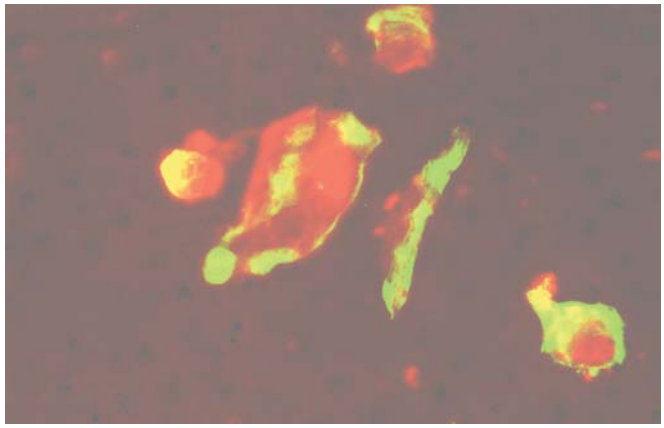

Fig. 2. Immunofluoresce of pul mesenchymal cells for C-kit (red). Cytoplastic actin is coloured in green.

The stem cells isolated from the pulp of deciduous teeth manifest a clear potential for spontaneous differentiation, which is shown by their coloured in red cytoplasm. This is indicative of the presence of the highly dissoluble SCF, which causes quick cellular growth. Its quantities increase with time and during the differentiation. The coloured in green actin found in the cells isolated from the pulp a deciduous tooth demonstrates the potential capability of the stem cells for differentiation to smooth-muscle. This is proven by the sustained and increasing with time positive SMA – smooth-muscle actin.

Nestin is a neurofilament which manifests itself in many different types of cells during the embryonal development. It is a neural progenitor, represents an intermediate filamentous structural protein that can be found in primitive neural tissue. Nestin is a structural protein that plays a main part in the legthening of the axons of the nerve cells. As the cells become more differentiated, its expression gets suppressed and it gets superseded by tissularly specific structural proteins. The expression of the nestin is widely used as a marker of the undifferentiated cells of the central nervous system.

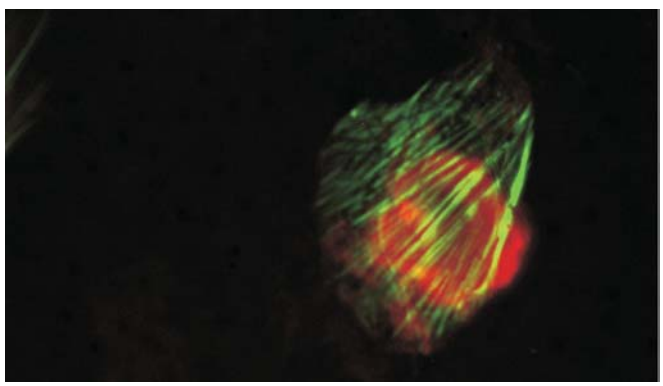

Fig. 3. Immunofluorescence of pulp mesenchymal cells for nestin (red). Cytoplastic actin is coloured in green.

The using of nestin as a marker for the differentiation of nerve cells shows that the cells from the pulp of deciduous teeth cultivated and stimulated by means of stem cellular factor demonstrate increased activity. This can be seen from the red pigmentation for nestin. From this fact we can deduce the conclusion that the differentiation of these cells is congenial with the differentiation during the embryonic development and dentinogenesis. Since the odontoblastic cells evolve from the neural combs and are in themselves ectomesenchyme, they have qualities not only of mesenchymal cells participating in the mineralisation of the dentine but also manifest some qualities typical of nerve cells. The reaction to nestin shows that the stem cells from the pulp of a deciduous tooth have the capability to differentiate to cells similar to the odontoblastic ones as well as to nerve cells. The hightened reactivity to nestin is indicative of the presence of cells with high pluriopotence. The simultaneous activity of differentiation to highly differentiated odontoblastic and nerve cells is a prove maybe of the combined function of the odontoblastic cells in the production of a hard mineralised structure, for its sustaining and for the manifestation of qualities similar to those of the nerve cells, as well as for the conduction of impulses. Probably the reactivity to nestine is an indispensable quality of the cells that are to differentiate to odontoblasts.

Simultaneously, the reactivity towards nestin demonstrates the multipotence of these cells, which when stimulated with appropriate growth factors and markers for nerve cells could undergo additional differentiation. The manifestation of an early marker for neurodifferentiation proves also the potential of SHED to get differentiated to nerve cells. This fact opens up new possibilities for research in the sphere of regenerative medicine.

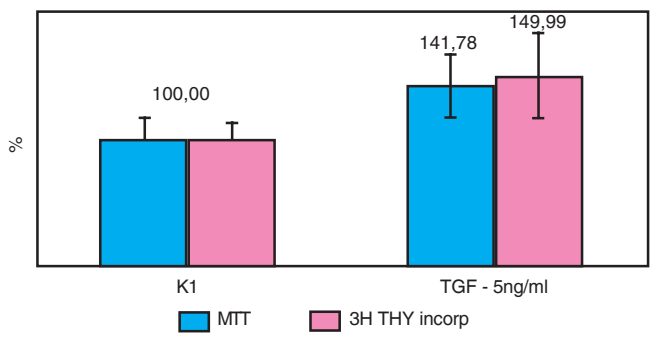

Fig. 4. Influence of TGF-в1 on the cellular proliferation of SHED

The addition into the nutrient medium of 5 ng/ml TGF-в1 causes an increase by 40% of the cell proliferation, compared to the cells grown in autocrinic conditions, during which a 50% increase of the MTT-activity in the cellular mitochondria is observed too.

The growth factor TGFв induces cell multiplication but cannot transform the cells from one type to another.

BMP-2 (Bone Morphogenic Protein-2) and TGF-в1 (Transforming Growth Factor-в1) are closelt related growth factors. They are members of the family of transforming growth factors.

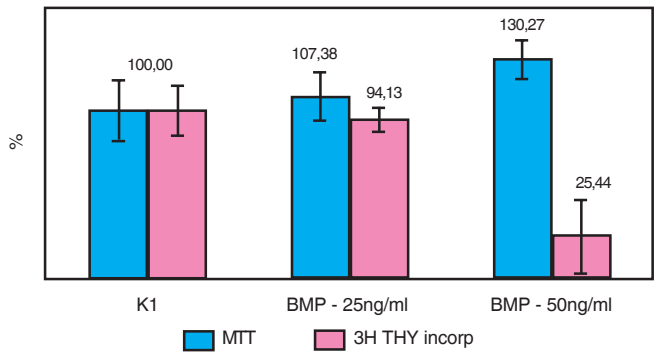

Fig. 5. The influence of BMP-2 on the proliferation and differentiation of SHED

The introduction of 25 mg/ml BMP-2 into the nutrient environment for 72h has a weakly manifested pro-differentiation impact. The synthesis of the DNA is reduced by 6% and the MTT-activity is increased by 7% compared to the autocrinically proliferating cell cultures. The introduction of 50 mg/ml BMP-2 into the nutrient medium for 72h has a strongly manifested pro-differentiation effect – the DNA synthesis is reduced considerably by 75% and the MTT-activity is increased by 30%. The increasing concentration of BMP-2 has a dose-dependant pro-differentiation effect, this leading to an increase in the survivability of the cells.

Even though the BMP is just one of the growth factors, it is a unique growth factor. It is the only morphogenic growth factor of all growth factors we know, capable pf transforming the connective-tissue cells into osteoprogenitory cells. Thus it is not only a mitogen stimulating the multiplication of the connective-tissue cells, but can also be a morphogen capable of transforming the connective cells into osteoprogenitory ones (20).

The action of the bone morphogenic proteins is the result of a complex transition of information through signal molecules. The bone morphogenic proteins connect to the cell surface through two types of receptor serine-trionine kinasic proteins (20). This causes the formation of heterotetrameric complexes of signal cascades. Through the consecutive phosphorilations of the kinasic receptors a transcriptional factor for the gene of the early form of BMP is activated. The mechanism is much more complex and in it participate signalisations from other growth factors too (20).

The similarity in terms of amino acid composition between BMP-2 and BMP-4 determines the similar effects they have on the development of the embryo. Such effects from BMP-2 and BMP-4 occur in the early stages of the embryonic development of the teeth too (11, 17, 20). Their concentration in the dental embryo during the bud-like stage increases and is retained at a high level during the whole development of the tooth embryo. These are factors leading to a differentiation of the mesenchymal and epithelim cells in the tooth embryo to odontoblasts and ameloblasts.

The fact that the stimulation of the stem cells isolated from the pulp of deciduous teeth by means of BMP-2 brings about the activation of their differentiation is indicative of the real potential for making use of these cells as well as for their potential for regenerative dental medicine. The link between the capacity for differentiation and the dose of the BMP-2 applied creates the possibility for a guided stimulation of the postnatal stem cells that are present in the pulp.

Generalisation:

The determined potential of postnatal stem cells in the pulp of deciduous teeth that are to be physiologically superseded creates new possibilities for obtaining and using stem cells in regenarative medicine. This is the most acceptable source of human postnatal stem cells and immediately after its discovery it turned into an important object of investigation. Stimulated by means of different growth factors, these cells evince a clear capability for proliferation and differentiation. The capability of these cells to evince a characteristic of early differentiation to different types of cells such as nerve cells, smooth-muscle cells, osteoblastic cells and odontoblastic cells is indicative of multipotence and even pluriopotence. In order that these qualities are proven the researches must develop and deepen both in terms of achieving a more advanced differentiation and in terms of making a comparison with the cells obtained from the pulp of grown-up individuals. The existence of a potential of stem cells in the pulp of a deciduous tooth creates a possibility for a leap in the the treatment of the early reversible phases of pulp inflammation. The stem cells stimulated through growth factors could build up an organic matrix and later remineralise it. This would facilitate the achievement of healing of the pulp-dentine lesions in a certain and physiological way. These stem cells could also be used in the regenerative treatment of the periodontal conditions, which would be a considerable progress in such treatment. The multipotent qualities of the stem cells can also be used as a proof of the capability of odontoblasts to react as nerve cells. And finally, if proven pluripotent, these stem cells could be used for the regeneration of other, non-dental, organs and tissues.

CONCLUSION:

The non-differentiated cellular elements in the pulp of deciduous teeth that correspond to norm do not evince a proliferation potential. Stimulated with growth factors they evince a manifested capabilty of proliferation, and later evince qualities of multipotent differentiation. Some signals of pluripotence are present, which requires further study. Researches must be deepened in the areas of late cellular differentiation and the capabilities for its application in regenerative therapy.

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