EVALUATION OF OSTEOGENIC ACTIVITY AND MINERALIZATION OF CULTURED CANINE PERIOSTEAL CELLS

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Summary

This study was made to evaluate "in vitro" the osteogenic potential and the mineralization capacity of subperiosteal stem cells on dog, comparing two isolation cells methods: from explant and by digestion. Periosteal fragments were detached from middle femoral diaphysis of eight common breed dogs. Half of the probes were used as explants and the other half were submitted to artificial digestion. The periosteal cells were than isolated, cultured and differentiated in DMEM environment. The osteogenic capacity evaluation of cellular cultures was made by alkaline phosphatase activity determination and the mineralization capacity of extracellular matrix was evaluated by Von Kossa reaction.

We have concluded that both periosteal cells isolation methods taken into study can be used for osteoprogenitor cells obtaining. In the presence of osteoinductive factors from the differentiation environment, the periosteal cells in the cultures start to differentiate in osteoblasts.

Key words: periosteal cells, osteogenic activity, mineralization,

Subperiosteal cellular layer contains stem cells capable to form lamellar bone tissue (4), helping in both diameter growth of cortical bones and in bone defects (fractures) healing.

Although on some laboratory animal species and humans, cultivation techniques are known and presented (1, 6, 9), these were less investigated on dogs (10).

In this study we have proposed to evaluate "in vitro" the osteogenic potential and the mineralization capacity of subperiosteal stem cells on dog, comparing two isolation methods: from explant and by digestion.

Materials and methods

The biological material used in this study was made by eight common breed dogs, both sexes, with ages between 3 and 5 years, weighting between 17 and 25 kg. Adapting in dog the technique described by Park et al. (7), under general anesthesia (acepromazine – ketamine - isoflurane), 10x20mm periosteal

fragments were detached from middle femoral diaphises of both femurs. Half of the prelevated flaps were sectioned in 10x5 mm fragments and used afterwards per se, being considered explants and the others were grinded and submitted to artificial digestion.

For the isolation of subperiosteal cells from explant and by artificial digestion was made according to the protocols presented by Declercq – 2005.

The cells obtained following isolation were than cultivated for six weeks. Dulbecco's Modified Eagle Medium (DMEM) cultivation environment that was used contains glutaMAX -1 + 10% bovine fetal serum + 0.5% penicillin streptomycin + 1 % Fungizone + 100µM L-ascorbic 2 phosphate acid. The cellular morphology was evaluated using contrast phase microscope at 4, 5, 8 and 14 days from the introduction of cells on cultivation environment. The determination of cellular viability was made by hemocitometric analysis of a suspension of cultivated cells stained with tripan blue (10 µl cellular suspensions and 50 µl tripan blue).

The cultivation on differentiation environments was followed-up for 10 days. On half probes, using environment osteoblasts and the other half was followed-up cultivated on DMEM.

The evaluation of osteogenic capacity of cellular cultures (those kept only in DMEM and those kept in DMEM and ascorbic acid, β glycerophosphate and dexamethasone -differentiation environment) was made by alkaline phosphatase activity determination (3). On both groups, the mineralization capacity of extracellular matrix was evaluated by Von Kossa reaction.

Results and discussions

After explant isolation method, the periosteal cells were identification after four days from beginning of cultivation (fig. 1). The isolation method by digestion permitted periosteal cells identification after four days from beginning of procedures (fig.2).

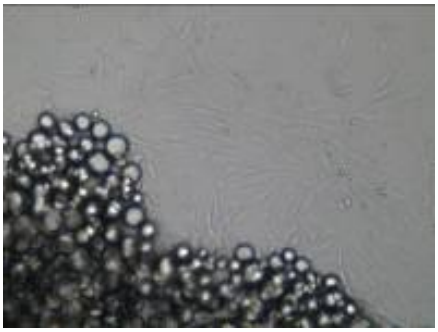

Fig. 1. Explants' aspect at four days from the insertion in cultivation environment (20X objective)

Fig.2. Periosteal cells isolated by digestion (20X objective)

The cultivation surface provided for the cells obtained by both isolation procedures was totally occupied till sixth day. The cellular morphology was similar to fibroblasts through all cultivation period both for cellular cultures resulted from explant and for those resulted from digestion. The cells are filiform and have a vortex disposition. In the cultivation disks, epithelial cells-like aggregations can be observed, those being constituted by independent fibroblastic cells grouped. These cellular aggregations are present in larger number in the cultures obtained from periosteal explants.

Both in periosteal cells obtained from explant and in those obtained from digestion, the cellular viability exceed 95% but the total number of the cells present on the same surface of cultivation varied in large limits between individuals – table 1.

Table 1

Cellular viability (cells number) after six weeks cultivation in DMEM

Subject	Cells no./ml cellular isolate from explant	Cells no./ml cellular isolate from digestion
1	4.5 millions	4.5 millions
2	1.8 millions	3 millions
3	10.5 millions	3.2 millions
4	3.2 millions	2.8 millions
5	6.5 millions	5.5 millions
5	2.4 millions	2.1 millions
7	7.5 millions	4.8 millions
8	4.2 millions	3.6 millions

On microscopically exam of the cells cultivated in DMEM, made after differentiation period, in which the cells were maintained in an osteogenic suspension (that contains ascorbic acid, β–glicerophosphate and dexamethasone), was observed that the cells have polyhedral shape and their multiplication in the culture environment was made mostly on vertical direction. These characteristics suggest a direct osteogenic differentiation of periosteal cells. Similar data was obtained by Groger et al. (5), using miniature pigs as experimental model and also by Uneo et al. (8) using guinea pigs as experimental models.

Periosteal cells cultivation in DMEM is a usual method for human cells (6). The use of this cultivation method for canine periosteal cells, leaded to a larger number of cells obtained and to an increased viability, fact that allows this method to be considered as adequate even for canine cells.

The results obtained at the reaction for alkaline phosphatase activity are direct correlated with the differentiation environment used. In case of the cells obtained from explant (control enclosures 1) and from digestion (control enclosures 2) but which were maintained in the differentiation period only in DMEM suspension (the cells are round or slightly spindle-shaped, morphological characteristics of fibroblasts that those cells had before their introduction into the differentiation

enclosures). Following the reaction for alkaline phosphatase activity, the cells were not stained and stain wrecks were observed into the spaces between cells which suggest the lack of phosphatase activity.

On microscopically examination of the lamellas on which periosteal cells obtained following digestion and maintained in suspension with osteoblasts environment in the differentiation period were fixed (differentiation enclosures 4), was observed that the shape of these cells is polihedric, approximately cuboids. Following the reaction for phosphatase activity the cells presented positive phosphatase activity, but were less stained that the ones obtained from explant and differentiated in the same environment (differentiation enclosures 3).

The positive activity of alkaline phosphatase, in case of differentiated cells suggests an intense osteogenic activity of periosteal cells of which differentiation was induced towards osteoblasts. Also, the morphology of these cells, polihedric, cuboids, of which growth was made even on vertical direction in the differentiation environment, is characteristic to osteoblasts.

The evaluation of mineralization capacity by von Kossa staining, revealed on lamellas examination (with explant cells – differentiation enclosures 3 and with cells obtained from digestion – differentiation enclosures 4, both maintained in osteoblasts differentiation environment) the presence of intense stained cells, umber nuclei, brown cytoplasm and the cellular membrane with adjacent area colored in black. The cells are polihedral. Around the cells, mineralized calcium deposal areas colored in black can be observed. This suggests a mineralization correlated with a positive osteogenic activity (3, 7).

On the lamellas with isolated cells from explant (control enclosures 1) and on those with cells from digestion (control enclosures 2), which were cultivated only in DMEM, it can be observed only an intense umber staining without calcium deposals. The lack of calcium deposals in the cellular membrane and around cells is characteristic to the lack of osteogenic activity (3, 7).

Conclusions

Both periosteal cells isolation methods taken into study can be used for osteoprogenitor cells obtaining.

Cultivation methods developed for human periosteal cells can be used for canine periosteal cells cultivation.

In the presence of osteoinductive factors from the differentiation environment, the periosteal cells in the cultures start to differentiate in osteoblasts.

Acknowledgments

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