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Skeletal Muscle-derived Fibroblast-like Cells Fail to Enable HeLa Cells to Induce Bone in the Murine Kidney

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HeLa cells fail to induce bone in murine kidneys, despite being highly chondro/osteogenic when implanted into thigh muscles. Bone induction in the kidneys failed also when HeLa cells were grafted together with skeletal-muscle-derived cell cultures. It is postulated that kidney parenchyma releases unidentified factcor(s) which prevent activation by inducer of cells termed Friedenstein's (1976) "inducible osteoprogenitor cells", while this hypothetical factor does not prevent further differentiation of "determined osteoprogenitor cells", thus allowing bone to form in the renal parenchyma.

Key words: Ectopic bone induction, kidney parenchyma, prevention of IOPC differentiation.

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Extraskeletal bone formation can be evoked by a variety of means, for example by implantation of living "inducers", such as uroepithelium (HUGGINS 1931), the epithelial established cell line HeLa, KB, WISH (ANDERSON & COULTER 1967; WŁODARSKI 1969, 1991) or by implantation of demineralized bone and tooth matrices (URIST 1965, 1971; URIST *et al.* 1968, 1969; for review see WŁODARSKI 1992).

Intramuscular injection of established epithelial HeLa, KB or WISH cell lines is followed by both endochondral and intramembranous ossification, and it is commonly accepted that implant-derived molecules, termed "osteoinductive factors" trigger the differentiation of implant bed resident mesenchymal cells toward the osteo/chondroprogenitor pathway.

These "osteoinductive molecules" belong to the bone morphogenetic protein family, are present in the bone and tooth matrices, and are synthesized by a number of neoplastic cell lines and by urothelium of several species.

Cartilage/bone formation by intramuscular implantation of HeLa cells is a model system for bone histogenesis research, is highly reproducible, and easy to perform. These cells, however, when implanted into kidney parenchyma failed to induce osteogenesis, despite good survival and proliferation of grafted cells (WŁODARSKI 1978). The reason why these cells are able to induce bone formation in skeletal muscle but fail to do so in the kidney is unclear. One possible cause is that the kidney lacks essential target cells, or target cells for some reason are unable to respond to the osteoinductive factors.

To verify the postulated lack in the kidney of target cells sensitive to bone-inducing principles, an attempt was made to introduce skeletal muscles-derived fibroblast into the kidney as a potential target cell for osteoinductive factors together with bone inducing cells, able to stimulate bone on intramuscular implantation.

Material and Methods

Established HeLa cell lines were cultured in standard MEM medium supplemented with 10% calf serum (Gibco), and harvested by trypsinization.

Muscle-derived fibroblast cells were cultured in the same medium. They were obtained by scissor-mincing of Balb/c female mouse abdominal muscles in the culture medium. The minced muscle chips were transferred into Falcon flasks and the outgrowing fibroblasts were propagated. After semiconfluence the muscle chips were removed by vigorous shaking and flask adherent fibroblast-like cells were further propagated and passaged using

0.5% trypsin solution aided by mechanical removal by policeman. The muscle-derived fibroblasts were harvested at various passage levels, ranging from 1st to 21st passage, suspended in the PBS solution and injected alone or together with HeLa cells in the proportion 1:1 of both types of cells.

Fibroblast alone or a mixture of fibroblasts and HeLa cells were suspended in the PBS solution. Three million cells of each type and a mixed injection containing 3 x 10^6 HeLa + 3x10^6 fibroblasts suspended in 0.1 ml of PBS, were injected into kidney parenchyma or under the kidney capsule. The kidneys were exposed after the dorsal skin was shaved and the skin and underlying dorsal muscles were open and right kidney was exposed. The wound was saturated with Ethicon 4/0 Vicryl rapide.

For surgery the animals were anaesthesized with chlorochydrate (0.1 ml of stock solution [360 mg/10 mL 0.9% NaCl]/10 g of body weight) given intraperitoneally. This procedure was approved by the Ethical Commission of Warsaw Medical University.

The bearers of kidney examined implants were also injected with 3x10^6 of HeLa cells into thigh muscles.

All animals were immunosuppressed by a single injection of 5 mg of Hydrocortisone (Polfa) administered under dorsal skin at the time of cell grafting.

The animals were killed 8-14 days later by cervical dislocation. The injected right kidney and the thigh muscles inoculated with HeLa cells were excised, fixed in Bouin's solution, demineralized in EDTA, and embedded in paraffin wax and serial haematoxylin-eosin-stained sections were examined.

The number of specimens examined, type and location of implants, and the appearance of mitotic figures as well as the presence of cartilage/bone in the examined samples are shown in Table 1.

Results

The intramuscular implantation of HeLa cells produced endochondral and intramembranous bone formation in (14/16) cases. Hyaline cartilage was observed in the vicinity of HeLa cells on the 8th day post grafting being gradually substituted by bone (Figs 1, 2). Mitotic figures were seen in the HeLa cells up to 2 weeks post implantation, indicating good survival of these xenogenic cells.

In contrast HeLa cells grafted into kidney parenchyma or under renal capsule, alone or mixed with the muscle-derived fibroblast-like cells did not induce cartilage/bone formation, regardless of the passage level of the fibroblasts. However, the HeLa cells showed mitotic activity in the kidney as they showed following intramuscular injection.

In no case did bone formation follow implantation of muscle-derived cells into either kidneys or muscles.

Table 1

Analysis of bone induction in murine kidneys and thigh muscles by HeLa cells alone, HeLa cells combined with muscle-derived cell cultures, and solely by muscle-derived cells at various passage levels

Implantation into kidney parenchyma or under kidney capsule			
Type of implanted cells	No of implants examined	No of specimens demonstrating mitotic figures	No of specimens demonstrating cartilage/bone presence
Muscle-derived fibroblast cell cultures (1 passage)	6	5	0
Muscle-derived cell cultures (2nd-21st passage)	6	4	0
HeLa cells	12	11	0
HeLa cels + fibroblasts (1-21st passage)	62	47	0
Implantation into thigh muscles			
HeLa	16	13	14
Muscle-derived cell cultures (1-21st passage)	8	2	0

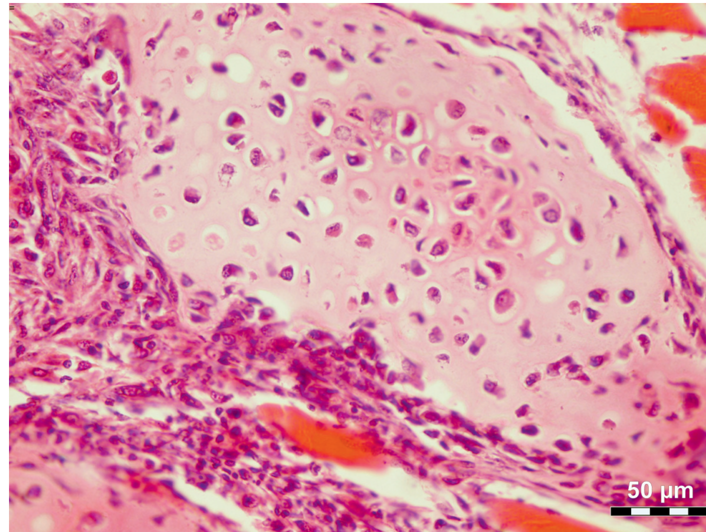

Fig. 1. Hyaline cartilage formation ten days following intramuscular implantation of HeLa cells. H-E staining.

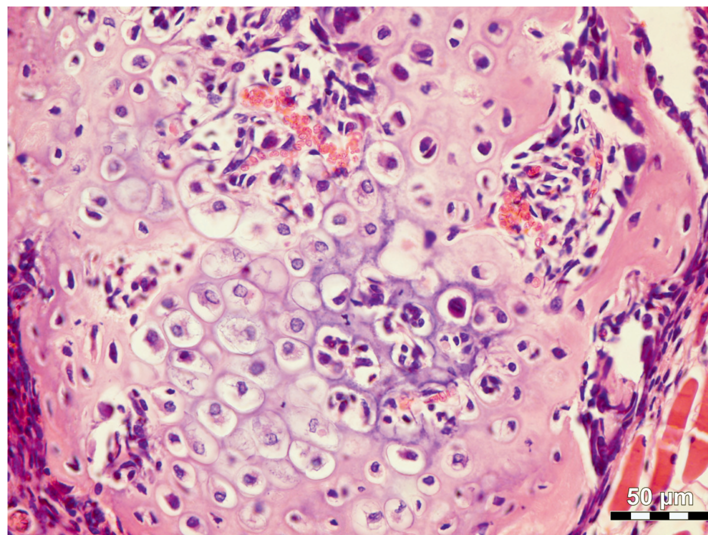

Fig. 2. Cartilage and bone induced by intramuscularly implanted HeLa cells, 18 days post grafting. The ingrowth of blood vessel into hypertrophied cartilage is an early manifestation of myelopoiesis inside the foci of heterotopic bone induction. H-E staining.

Discussion

In humans bone formation in the kidney is an unusual occurrence. Case reports relate bone formation in the kidney to trauma (HOLMES & BASKIN 2001), renal cell carcinoma (MORIYMA *et al*. 2002) and poorly differentiated transitional cell carcinoma (TAWADA *et al*. 2001).

We were unable to obtain bone induction in the kidney by either HeLa cells grafted alone or mixed with skeletal muscle-derived cell cultures, whereas HeLa cells induced bone in a high proportion of animals following injection into thigh muscle.

In a pilot experiment demineralized tooth matrices implanted into kidney parenchyma also failed to induce osteogenesis, but were potently osteogenic when implanted into tight muscles (data not shown).

In this paper we confirm the earlier observation of failure of bone induction in the kidney by agents which induce bone following introduction into skeletal muscles (WŁODARSKI 1978; URIST *et al.* 1969).

On the basis of the results, we postulate that the kidney environment exerts an inhibitory effect on differentiation of osteoinducible progenitor cells such as skeletal muscle-derived cells (FRIEDENSTEIN 1976). In kidneys the morphogenetic signal trigged by HeLa cells as well as released from a demineralized tooth matrix does not stimulate osteogenic differentiation of either kidney paren-

chyma resident fibroblasts or muscle-derived fibroblast-like cells.

The kidney environment, however, does not prevent formation of bone from determined osteoprogenitor cells (DOPC) such as osteoblasts or bone marrow stromal cells. Bone histogenesis is easily obtained in the kidneys by implantation of bone marrow derived stromal cells or whole bone-marrow cell suspensions containing DOPC (VARAS *et al*. 200; WŁODARSKI *et al*. 2004, 2006).

Thus it is postulated that kidney parenchyma inhibits differentiation of inducible osteoprogenitor cells (IOPC), such as muscle-derived fibroblasts, but not determined osteoprogenitor cells (DOPC), such as bone marrow stromal cells. Because of this property the kidney, as a vital organ, could be protected against bone induction.

There is a long list of inducible osteoprogenitor cells, osteoprogenitors for bone inducing agents, among these pericytes, endothelium, thymus cells, peritoneal fluid cells and mesenchymal cells (BURING 1975; FRIEDENSTEIN 1976). However, in endothelium and pericyte-rich tissues such as kidney, liver and spleen, bone induction fails (URIST *et al*. 1969). CHALMERS *et al*. (1975) demonstrated that after transplantation of an induction site from skeletal muscle into the kidney, ectopic osteogenesis ensued. This phenomenon is best explained by hypothesizing that during the time the inducer spent in skeletal muscle, IOPCs were converted into DOPCs, and on retransplantation were therefore able to form bone in the renal milieou which appears to inhibit any further differentiation of either renal or, as in the present work, introduced IOPCs.

The kidney parenchyma is unable to respond to osteoinductive stimuli. The introduction of IOPCs, such as native mesenchymal cells resident in the muscles, into the kidney does not cause osteogenic differentiation. Thus a possible explanation for extremely rare osteoinduction in humans and lack thereof in mice, but not of further differentiation of previously determined preosteoblast, is the prevention of activation of IOPCs (of both kidney residents or introductions) by unidentified factor(s) released by the kidney parenchyma.

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