Time course of the regeneration of the endplate zone after autologous muscle transplantation

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Summary. Reinnervation of transplanted muscles occurs in 2 steps. During the first 5 months the motor axon terminals innervate primarily the border of the original endplate zone, re-establishing its previous outline. In the next 4 months, by further ramifying of the axons, new nerve-muscle contacts are formed exclusively within the boundaries of this zone.
Key words. Muscle transplantation; motor endplates; endplate zone; regeneration.

The postoperative time course of degenerative and regenerative events in transplanted muscles is well documented[1-8]. However, long-term studies of the regeneration of the endplate zone (EZ), i.e. the area containing motor endplates (EPs) have not yet been made.

Material and methods. All transplantations were carried out on young (8–9 weeks) male Wistar rats (strain WU, Ivanovas GmbH, Kisslegg i.A., FRG). Under anesthesia with Hypnorm (1.5 ml/kg b.wt i.m.) in combination with Valium (2.5 ml/kg b.wt i.p.), the left superior sternohyoid muscle was excised and sutured back into its anatomical position. The nerve stump(s) were loosely sutured to the original place of nerve entry. The contralateral muscle was left untouched and served for comparison. After survival times of 1, 2, 3, 6, 8, 10, 16, 24, 30, 36 and 52 weeks, respectively, 3–4 animals per stage were killed by an overdose of Nembutal. Non-operated time-matched animals served as controls. The left and right superior sternohyoid muscles were removed 'en bloc' and immediately frozen in isopentane cooled by liquid nitrogen (−160 °C). Serial cryocut cross sections (12–14 µm) were processed for the combined demonstration of myofibrillar ATPase (after alkali and acid preincubation) and acetylcholinesterase (AChE) according to Ashmore et al.[9] and Toop[10]. The measurements were performed on longitudinal cryocut sections (40 µm) of control animals. They were treated according to the α-naphthylacetate method for non-specific esterases with fast red as a coupling agent[11]. Fiber typing and counting was performed as described earlier[12]. The number of EPs was determined semiquantitatively by counting them in every 7th cross section. The fields containing EPs (fig. 1) in these sections were graphically recorded along the longitudinal axis of the muscle (fig. 2).

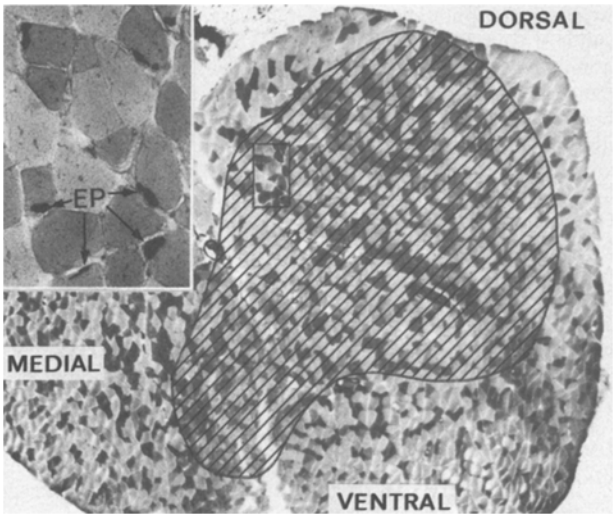

Figure 1. Cross section of a control superior sternohyoid muscle stained for myofibrillar ATPase (preincubation at pH 10.5, incubation at pH 9.5) and AChE. The field containing EPs is hatched. × 23.5. Inset: higher magnification of the rectangular area displaying the different muscle fiber types (type IIA intermediately, type IIB lightly stained) and motor endplates (EP). Four of them are marked with arrows. ×108.5.

Results. The superior sternohyoid muscle of control rats consisted of 3050±150 (SD) muscle fibers each exhibiting one single EP (as determined in preliminary experiments). With our semiquantitative method 30–35% of all EPs were counted. This percentage varied only slightly during the main period of observation (from 16 weeks of life onward) since the EPs barely showed further growth. Until 3 weeks after transplantation no EPs were detectable in the regenerating muscles. At 10 weeks postoperatively (p.o.) the number of *EPs* displaying AChE activity reached approximately 50% of the original amount; the percentage increased to 90–95% at 36 weeks p.o. (the final value). Independent counting of reinnervated muscle fibers showed the same percentage. The combined staining for ATPase and AChE did not allow fiber typing before nine months p.o., i.e. only after completion of the reinnervation. Thus an eventual selectivity of the reinnervation process with regard to a distinct fiber type could not be investigated with this method. The *EZs* of the grafted muscles regain their original size (corresponding to the size of the contralateral one) already after 20 weeks p.o. (table). At all stages investigated the size of the EZ was the same in contralateral and control muscles.

Discussion. In the transplanted superior sternohyoid muscle active EPs (determined by their histochemical AChE activity) first appear after 3 weeks. Their number increases up to 9 months after surgery and this increase is accompanied by an increase in

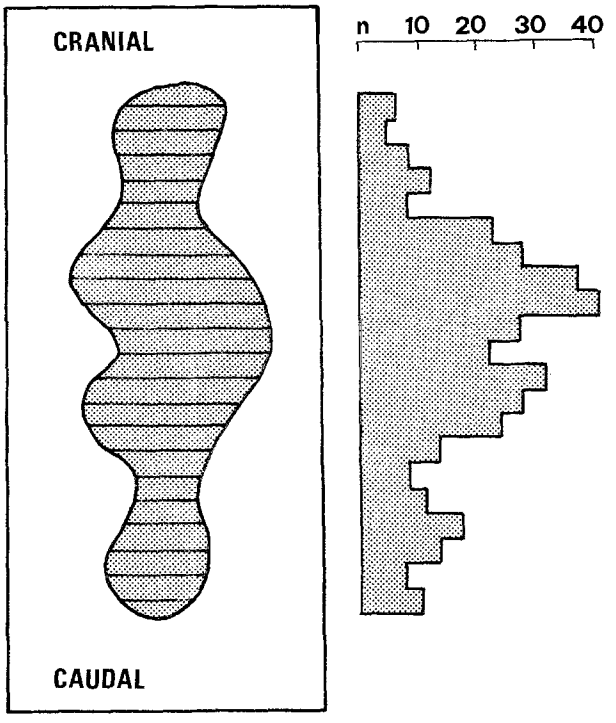

Figure 2. Frontal projection of the EZ in a superior sternohyoid muscle, 6 months after transplantation. The horizontal lines represent the width (medial-lateral) and the relative position of the fields containing EPs in every 7th section. The histiogram on the right indicates the number (n) of EPs in the corresponding sections.

the amount of reinnervated muscle fibers. The EZ, however, gains its full original size and shape already after 5 months, i.e. 4 months before reinnervation density is completely restored. Therefore it must be assumed that in a first step the reinnervating motor axon terminals mainly contact the original margin of the EZ. In a second step EPs proliferate exclusively between the borders of the re-established EZ by further ramifying and sprouting of the motor axons. With the methods used it cannot be decided whether the EPs in the transplanted muscle are newly formed or, rather, old ones. However, old EPs can persist for several months in necrotic tissue[1,13]. This fact, together with the finding of the regenerated EZ within its original limits [own observation and previous findings[5,14]] supports the second assumption.

Age (weeks)	3	6	10	16	24	30	36
nEP_T/nEP_C	0.23	0.33	0.71	0.72	0.84	0.88	0.97
lEZ_T/lEZ_C	0.38	0.52	0.74	0.86	1.02	0.95	1.05

Ratios of the mean values of the number (n) of EPs and the length (l) of EZ between the transplanted (nEP_T; lEZ_T) and the contralateral (nEP_C; lEZ_C) muscle. This kind of data presentation eliminates interindividual differences. Preliminary investigations have shown that the EZs of the two superior sternohyoid muscles in the same animal are highly symmetrical in length and position and the number of muscle fibers is virtually identical. This allows the comparison of the number of EPs and the size of the EZ between the transplanted and the contralateral muscle within the same animal.

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Effect of halogenmethylenebisphosphonates on bone cells in culture and on bone resorption in vivo

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Summary. Dihalogenmethylenebisphosphonates increase alkaline phosphatase activity and fatty acid oxidation in calvaria cells in culture ($Cl_2MBP > Br_2MBP \cong F_2MBP$). The monohalogen ClMBP and the non-halogenated analogues are less active on phosphatase and inactive on or inhibitory towards fatty acid oxidation. The three dihalogenbisphosphonates and ClMBP inhibit bone resorption in vivo, Cl_2MBP most strongly.
Key words. Bone cells; bisphosphonates; lactate production; alkaline phosphatase; fatty acid oxidation; bone resorption.

Bisphosphonates are analogues of pyrophosphate with a P-C-P bond instead of a P-O-P bond. In vitro they inhibit precipitation and dissolution of calcium phosphate[2-5], and in vivo they slow down bone resorption[3,5-8] and decrease ectopic calcification[2,4] and to some degree bone and cartilage mineralization[6]. While the effect on mineralization appears to be due to a physical chemical action on crystal growth, the mode of action on bone resorption is still unclear. Thus, no correlation has been found between the effect of different bisphosphonates on crystal dissolution in vitro and bone resorption in vivo[9], and because the amounts active in vivo are so small, an action on crystals is unlikely. Consequently, in contrast to mineralization, the action of bisphosphonates on bone resorption is likely to be cellular. Indeed, studies in cell culture have shown that bisphosphonates are taken up by bone cells[10,11] and that they affect various parameters such as cell number[11], production of lactic acid[11], alkaline phosphatase activity[12], fatty acid oxidation[13], collagen[14] and proteoglycan[15] synthesis, PGE_2 production[16] and resorption of bone particles by macrophages[17]. The relevance of these effects of bisphosphonates in cell culture to the inhibitory action of bone resorption is, however, still unclear.

One of the bisphosphonates which has been studied most extensively is Cl_2MBP (for abbreviation of bisphosphonates see table 1). It is a potent inhibitor of bone resorption, but has only a small effect on bone mineralization, both in animals[6] and in humans[18-21]. In vitro Cl_2MBP has the ability to dramatically increase alkaline phosphatase activity of bone cells[12], a property which is shared, although to a lesser degree, by another dihalogen derivative Br_2MBP[9]. Furthermore, Cl_2MBP increases the oxidation of fatty acids[13]. Since these two effects have not been observed with any other of the bisphosphonates investigated up to now, they might be a special characteristic of dihalogenmethylenebisphosphonates. In order to test this hypothesis, the action of Cl_2MBP, Br_2MBP, and F_2MBP as well as that of the monohalogen ClMBP and of nonhalogenated structural analogues have now been investigated.

Since all three dihalogenbisphosphonates have been shown to inhibit bone resorption either in vitro[9,22] or in vivo[9,23], they were compared one with the other, and also with the monohalogen derivative ClMBP, on their effect on bone resorption in vivo. This was carried out in order to assess a possible quantitative relation between their activity on bone resorption and on cell metabolism.

Material and methods. Cell culture. Calvaria from 1-day-old Wistar rats were digested with collagenase. The cells, which included osteoblast-like, periosteal and fibroblast-like cells, but no multinuclear osteoclasts, were then cultured in minimal essential medium containing Earle's salt solution and 10% fetal

Table 1. Bisphosphonates tested

Abbreviations	Full name
Cl_2MBP	Dichloromethylenebisphosphonate
Br_2MBP	Dibromomethylenebisphosphonate
F_2MBP	Difluoromethylenebisphosphonate
ClMBP	Chloromethylenebisphosphonate
MBP	Methylenebisphosphonate
HMBP	Hydroxymethylenebisphosphonate
HEBP	1-Hydroxyethylidene-1,1-bisphosphonate