Differentiating Limb Tissue Affects Neurite Growth in Spinal Cord Cultures

Abstract. *Limb bud mesenchyme enhances and directs the growth of tadpole spinal cord nerve fibers in tissue culture. This effect on elongating neurites may involve alterations in nerve-substratum interactions by the presence of undifferentiated target tissues. The relationship between nerve fibers and their potential innervation sites can explain directed nerve growth to the developing limb.*

During vertebrate development, nerve fibers leave the motor areas of the spinal cord and extend to their peripheral target tissue, the limb bud, prior to differentiation of the limb musculature (*1*). We have undertaken an examination of the influence that the differentiating limb may have on the nerve fibers emanating from the central nervous system (CNS) in a tissue culture model. Undifferentiated limb mesenchyme and partially differentiated limb tissue appear to exert a significant influence on the growth of nerve fibers from larval frog spinal cord explants in vitro (*2*) in the form of enhanced neuritic outgrowth and neurite orientation (*3*) in the direction of the peripheral tissue. We have thus postulated that a product of limb mesenchyme enhances the growth and orientation of nerve fibers from the developing spinal cord. The characteristics of this growth suggest that the adhesive relationships between nerve fibers and the substratum are altered in the presence of mesenchyme so as to form a pathway of preferred nerve adherence in a gradient-like fashion.

The amphibian tadpole is a useful model for this study since spinal cord and limb explants can be maintained for extended periods in a serum-free, defined medium so that the permutations and masking of effects due to the presence of serum can be avoided. In addition, the relatively protracted rate of tadpole development ensures an adequate period for stage-dependent interactions. Cross-sectional segments (~ 0.5 mm^3) of meninges- and ganglia-free lumbosacral spinal cord and epidermis-free hind limb of *Rana pipiens* tadpoles [staged by the Taylor-Kollros larval series (*4*)] were explanted onto collagen-coated cover glasses and sealed into Maximow depression slide assemblies with nutrient medium by methods established in our laboratory (*5*). Limb explants were either added to the cultures concurrently with the cord explant or added to the cord-containing cultures after the establishment of neuritic outgrowth, as outlined in Table 1. Cultures of cord explants alone served as controls. All cultures were incubated at 19°C with medium renewal twice per week. A total of 96 cultures were examined and photographed frequently through an inverted microscope with differential interference contrast optics. The occurrence of neurite growth enhancement in the experimental arrays was determined by comparing them with same-stage controls after both tissues were in culture for 10 to 14 days. Because of the nonradial, complex nature of neuritic outgrowth (see below) from the spinal cord explants, quantification was restricted to comparative scoring. Nerve fibers were considered to have oriented growth if they curved or branched significantly in the direction of the peripheral tissue. Very dense outgrowth only from the cord region nearest the limb tissue was also indicative of a directional effect.

Control spinal cord explants from stage V and stage IX tadpoles exhibited sparse, randomly directed neuritic outgrowth with characteristically straight fibers. Although they often had associated symmetrically arranged branches, these neurites rarely curved. The distal portions of the nerve fibers were undistinguished and had typical nerve growth cones (*6*). In general, the more mature stage IX control spinal cord explants possessed sparser outgrowth than the younger stage V ones. These features were dramatically altered in the presence of limb tissue placed as far as 2.0 mm (usually 0.5 to 1.5 mm) from the spinal cord explant (Fig. 1). Observable effects were reduced or absent when the distance between the explants greatly exceeded 2.0 mm. The results obtained with the various culture combinations are summarized in Table 1.

Stage V limb tissue, predominantly mesenchyme, greatly enhanced the extent of neuritic outgrowth, regardless of when it was added to the stage V cord cultures. In these cases, the nerve fibers often oriented in the direction of the limb tissue. The increased density of the outgrowth from the cord edge nearest the limb was a result of both augmented branching of neurites and the frequent occurrence of complex, distal collateral arborization.

Nerve fiber length was usually greater for experimental than for control cultures, but the presence of fascicled fiber

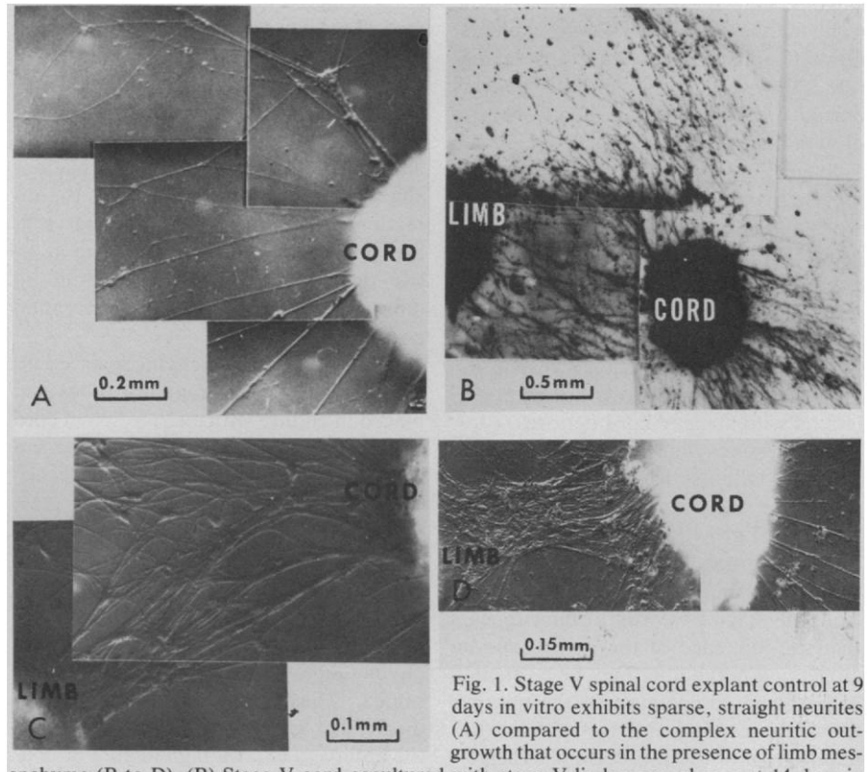

Fig. 1. Stage V spinal cord explant control at 9 days in vitro exhibits sparse, straight neurites (A) compared to the complex neuritic outgrowth that occurs in the presence of limb mesenchyme (B to D). (B) Stage V cord cocultured with stage V limb mesenchyme at 14 days in vitro has dense, arborizing outgrowth. (C) Stage V cord cocultured with stage V limb mesenchyme at 11 days in vitro directs its curving neurites to the limb tissue. (D) Stage IX cord cocultured with stage V limb tissue at 15 days in vitro has very dense neuritic outgrowth on side nearest the mesenchyme and predominantly straight, sparse neurites on the opposite side.

Table 1. Mean relative growth response of tadpole spinal cord neurites to the presence of various peripheral tissues in culture. Abbreviations: C, concurrent explanting of cord and peripheral tissue into the same culture; L, late addition of peripheral tissue to established cord cultures of 9 to 14 days; 0, control equivalent with random outgrowth; +, enhanced neuritic outgrowth compared to controls, or definitive orientation of many neurites toward peripheral tissue; ++, maximally enhanced neuritic outgrowth, or orientation of most neurites toward peripheral tissue; −, less neuritic outgrowth than controls, or repulsion of neurites away from peripheral tissue.

Spinal cord stage and response	Peripheral tissue (stage)									
	Limb (V)		Limb (IX)		Limb (XI)	Muscle	Heart		Liver	
	C	L	C	L	L	L	C	L	C	L
Stage V cord										
Outgrowth response	++	++	0	+	0	++	−	−	−	0
Direction response	+	+	++	++	0	0	−	−	0	0
Stage IX cord										
Outgrowth response	+	++	+	++	++	++				
Direction response	+	+	+	+	0	0				

bundles was variable. The alterations in the growth of the neurites were those to be expected from an increase in the adhesiveness between the nerve fiber and the substratum (*7*). Similarly, the degree of fasciculation may be a function of nerve-to-nerve adherence (*8*), and a competition between it and nerve-substratum adhesion could account for the variations in this respect. Directed outgrowth toward the limb tissue was most often seen as a curving of the elongating fibers in that direction as well as a preferred branching toward the limb. This stage V limb mesenchyme acted in the same manner on stage IX cord explants. Growth enhancement was most elaborate, however, if the limb tissue was placed in culture after some neurite growth had taken place.

Stage IX limb explants, composed of mesenchyme, muscle, and cartilage, were effective in eliciting directed growth from stage V cords, but less so in enhancing the density of neuritic outgrowth. This limb tissue also promoted neurite growth from stage IX spinal cords as well as directional responses. However, orientation was not as prevalent as with stage V cord neurites.

Nonmesenchymal limb explants from stage XI tadpoles had no effect on neurites from stage V cords, nor on the orientation of growth from stage IX cords. Yet, this largely differentiated tissue did elicit significantly enhanced outgrowth from the stage IX cords. It thus appears that the influence of the limb tissue on elongating nerve fiber may be a function of both the competence of the nervous tissue to respond and the state of differentiation of the target tissue. Clearly, the least differentiated limb tissue, the mesenchyme, was most effective in evoking growth responses.

Mature limb muscle fragments also enhanced neuritic outgrowth to a great extent from both stage V and stage IX spinal cords, but without any indication of a directional influence. The difference between the response to a stage XI limb explant and the response to mature muscle fibers may lie in the regeneration processes that the muscle underwent in these cultures. The many similarities between limb regeneration and normal development, especially with respect to biochemical properties, support this contention (*9*).

It is noteworthy that stage IX spinal cord outgrowth responded most effectively when the limb tissue was added to the culture after neuritic outgrowth was under way. Some enhancement of outgrowth also occurred for stage V cords with the later addition of stage IX limb explants. These temporal relationships did not significantly affect the direction of neurite growth. The nerve fiber may acquire properties during in vitro development that can result in differential responsivity to substratum conditons.

Stage V limb mesenchyme inserted into borosilicate glass capillary tubes and placed in culture with stage V spinal cord explants did not prevent either the enhancement of outgrowth or neurite orientation. The enhanced outgrowth grew toward the open ends of the tubes, avoiding the most direct route from the cord to the tube center. Cord explants did not exhibit any effect due to the presence of an empty tube or of mesenchyme-filled tubes that had their ends sealed. These experiments further indicate that limb mesenchyme may have the ability to exert a high degree of control on nerve fiber growth, most likely by altering the substratum along which the nerve outgrowth seeks its target tissue.

The specificity of the influence of limb mesenchyme on nerve growth was tested by utilizing stage V spinal cord explants cocultured with stage V heart and liver tissues. Neither of these tissues exerted any positive growth influence on the neurites. Heart tissue, in fact, reduced the outgrowth and caused nerve fibers to avoid the heart region of the culture. It is pertinent that the normal innervation of the heart and liver is by means of the autonomic nervous system, as contrasted with CNS innervation of the limb.

Even though there have been numerous reports concerning the relationships between the developing CNS and the periphery (*10*), they have been without comment on possible mesenchymal influences. That a target organ is capable of stimulating nerve growth and direction has been demonstrated for autonomic nerves and their target tissues (*11*). In view of the results reported here and information on the early growth of nerve fibers through extracellular space from the CNS to the mesenchymal limb bud in situ (*12*), we suggest that nerve fibers first approach the limb target by means of a substratum pathway that is predetermined to some extent by a product of peripheral mesenchyme.

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References and Notes

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