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Quantitative Analysis of Vascularization and Cytochrome Oxidase Following Fetal Transplantation in the Contused Rat Spinal Cord

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ABSTRACT

In the normal adult central nervous system, a coupling between energy consumption and vascular density is well established. Likewise, the survival of fetal neural tissue grafts is highly dependent on the establishment of functional vascular integration with the host. However, to what degree graft vascularization and tissue metabolism influence the normal host response to traumatic injury has not been extensively studied. In the present report, embryonic day 14 fetal spinal cord suspension grafts were made into the lesion epicenter of subchronic (10 days) contusion-injured rats. Three months later, intraspinal transplants were analyzed using correlative cytochrome oxidase histochemistry and vascular morphometric analysis. The same approaches were applied to the host spinal cord and injured, non-transplanted animals in order to determine the ability of a graft to alter the level of post-injury vascularization and/or metabolism. In general, graft vascular density was increased over that measured in normal or injured gray matter. Vascular density in gray matter near the host/graft interface was markedly increased when compared to either gray matter of the same spinal level in injured non-grafted animals or normal control spinal gray matter. Vascular changes were not noted in gray matter 3 mm distal to the lesion epicenter (rostral or caudal) in all groups analyzed. Cytochrome oxidase was up-regulated at this time in the graft and gray matter at the host/graft interfaces when compared to either gray matter of the same spinal level in injured, non-grafted animals or that of uninjured controls. These data indicate that an intraspinal transplant placed into the contused adult rat spinal cord reaches a metabolic capacity that is likely to be associated with high levels of oxidative metabolism in the well-vascularized graft neuropil. In addition, transplantation chronically alters vascularization and metabolic patterns of adjacent spinal gray matter following contusion injury.

Indexing terms: angiogenesis, graft, metabolism, spinal cord injury, image analysis

Angiogenesis in graft tissue is a major factor in fetal graft survival and development (Bjorklund et al., 1983; Lawrence et al., 1984; Broadwell et al., 1990). Whole tissue, and to a lesser degree suspension grafts, are dependent on an early, host-derived angiogenic response. Graft size and integration are tightly coupled to the emergence of endothelial sprouts at the host/graft interface and subsequent vascular perfusion (Krum and Rosenstein, 1988). In any transplant paradigm, however, grafts must endure a period of ischemia during tissue preparation and during the acute post-transplantation interval. While angiogenesis is important for graft survival, it is unclear whether the vasculature of mature transplants is regulated by the same physiological factors (e.g., metabolic rate) that determine microvascular density in normal adult gray matter (Adair et al., 1990). For instance, an imbalance in this normal homeostatic environment could result in luxury perfusion or tissue ischemia in the normal brain. Although such a scenario has not been described with regard to fetal transplants, even subtle disparities between graft metabolic need and tissue vascular supply may account for reduced growth and survival of transplants placed into the brain (Krum and Rosenstein, 1988). The degree of mismatch may be one factor which

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dictates the metabolic potential of mature transplants. In this regard, site-specific fetal grafts have been described with normal metabolic rates (Kelly et al., 1985) and metabolic rates significantly lower than neighboring host tissue (Sharp and Gonzalez, 1984).

In the present study, a characterization of vascularization and metabolism of matured 3-month fetal grafts placed into the contusively injured spinal cord was conducted. Computer-assisted morphometric analysis of blood vessels was used to examine the vascularization of graft tissue, as well as neighboring and anatomically distant host spinal gray matter. In addition, gray matter at similar spinal segments was analyzed in injured, non-grafted and control uninjured animals. To determine whether a relationship exists between graft metabolism and vascular growth, a histochemical analysis for the presence of the metabolic enzyme cytochrome oxidase (COx) was also performed in similar spinal regions and experimental groups. It has been established that the mitochondrial density of this enzyme can be used as an index of neuronal metabolic rate both in the central nervous system (Wong-Riley, 1989; Di Rocco et al., 1989) and fetal grafts (Mufson et al., 1987; Dyson et al., 1988). In addition, a direct correlation has been established in the adult spinal cord between the cellular concentration of COx and local metabolic demand (Wong-Riley and Kageyama, 1986; van Raamsdonk et al., 1987).

The present data suggest that well-integrated transplants in the contused spinal cord are highly metabolically active and correspondingly well vascularized. In addition, the presence of a fetal graft alters the metabolic and vascular patterns of the contused spinal cord. Together, the findings indicate a potential level of functional integration that has not been previously reported.

MATERIALS AND METHODS

Animals and injuries

Adult female HARLAN Sprague-Dawley (n = 62) rats ranging between 250 and 300 g were used for these studies. Vascular density measurements were performed in control uninjured rats (n = 6), animals receiving a contusion injury and spinal transplant (n = 26), or a contusion injury alone (n = 12). Similarly, a quantitative assessment of cytochrome oxidase histochemistry was performed in control uninjured rats (n = 4), animals receiving a contusion injury and spinal transplant (n = 8), or a contusion injury alone (n = 6). All animals were anesthetized with an i.p. bolus of ketamine HCl (80 mg/kg) and xylazine (10 mg/kg). Contusions were then performed as previously described (Stokes, 1992; Stokes and Reier, 1992). Briefly, animals received a rapid contusion injury through an exposed laminectomy site at the level of the 8th thoracic vertebra. The dura was slightly compressed to a known starting point (3,000 Kdynes). A peak compression of 0.9 mm over a 23 millisecond epoch was subsequently achieved. The force, acceleration, and displacement of the impact probe are continuously monitored during the injury process. The reliability of such parameters in predicting sequelae of the injury has been previously reported (Behrmann et al., 1992).

All injury sites were covered with Durafilm to minimize connective tissue adhesions on the dura. Muscle and skin wounds were then sutured in layers. After surgery, the animals were placed on a heating pad and allowed to recover spontaneously. They were subsequently given fluids to maintain hydration and allowed to eat ad libitum. Bladders were expressed three times daily until voiding reflexes returned (usually within 2 weeks). Antibiotics (4.0 mg Gentocin, i.p.) were administered until the return of bladder function. In addition, vitamin C supplements (10 mg/day) were given to all animals as they were entered into the study and continued until the end of the experiment.

Transplantation procedures

Transplantation was performed at 10 days post-injury (n = 34). Whole segments of 14-day fetal spinal cord (approximately 8–10 cords) were prepared as previously described (Stokes and Reier, 1992). Solid tissue pieces were then transferred to ice-cold 0.6% glucose-saline, minced into small fragments, and transferred to a microcentrifuge tube. Total fluid volume was adjusted to approximately 400 μl. Tissue was dissociated by trituration using silicone-coated glass pipettes of progressively decreasing tip diameter with final trituration through a Model 701 (25 μl) Hamilton syringe. As before, cell viability and density were assessed immediately and after performing the last cell injection of the day (Stokes and Reier, 1992). After the cell suspension was prepared, the animals were anesthetized and the laminectomy site re-exposed and cleaned. The tapered needle of a 27-gauge Hamilton syringe was then inserted through the intact dura into the lesion epicenter and a total of 40–60 μl of the suspension injected. Injured-controls received glucose-saline vehicle injections only.

Quantitation of vascularization

Under anesthesia (described above), animals underwent intra-cardiac perfusion with 4% paraformaldahyde/2.5% glutaraldehyde. Spinal cords were removed and postfixed in the double aldehyde solution for an additional 24 hours. Spinal cords for quantitative vascular assessment were then cut into 1 mm blocks and embedded in Epon. Two micron, semi-thin sections were cut, mounted on slides, and stained with toluidine blue. Blood vessels were quantitated by visualization through a Dage Model 72 video camera interfaced with a Zeiss Axiophot microscope connected to a VIDAS image analysis system (Zeiss Inc.). In this preparation, graft vessels are arranged in a random or tortuous fashion (Horner et al., 1991) similar to that observed in other suspension transplant paradigms (Broadwell et al., 1991). As a result, accurate determinations of various morphological parameters can be obtained from a random plane of section. Blood vessels in normal spinal cord gray matter, however, are arranged longitudinally. This is especially true of thoracic spinal levels that are supplied by few radicular arteries and travel long rostral to caudal distances parallel to the spinal cord's longitudinal axis (Tveten, 1976). Unlike the randomly oriented graft vasculature, normal spinal vasculature therefore represents an anisotropic structure (e.g., cutting the cord in cross section produces fewer longitudinal profiles than a horizontal section). Consequently, the plane of section and quantitative parameters must be carefully chosen. In the present experiments, error due to anisotropy was reduced by using semi-thin sections which limit the number of longitudinally cut vessels and by choosing descriptive measures which are minimally affected by stereology or can be accurately adjusted.

Three parameters were chosen for quantitation: 1) surface fraction of blood vessels, 2) average blood vessel diameter, and 3) average blood vessel number. The first, surface fraction (area occupied by vascular profiles/area of

the measuring frame) is an estimate of vascular volume and is minimally affected by vessel orientation. Surface fraction does not differ in sections cut horizontally or in the coronal plane as the angle of section affects both the profile area and the containing space (Weibel, 1979). Using the VIDAS system, vessels viewed in a 100 μm^2 frame can be automatically selected based on the low shading level of their lumen. In this way a narrow band of gray levels can be used as a criteria for discriminating blood vessels from surrounding structures. It was therefore possible to discriminate consistently between blood vessels and myelin profiles. Selected vessels also were confirmed by microscopic examination. Profiles were excluded if they had a diameter larger than 14 μm, were not associated with an endothelial cell, or contained vascular smooth muscle. A blood vessel surface fraction was then calculated (surface fraction = surface area of blood vessel/surface area of the measuring frame) based on the adjusted image. The second parameter, blood vessel diameter, was also calculated from the previously collected images. The VIDAS assessed a circularity factor for each blood vessel profile (minimum diameter/maximum diameter). In this way only vessels that were cut transversely (e.g., no longitudinal orientation) were used to calculate average diameter (e.g., vessels containing a circularity factor of 0.9 or greater were included). This resulted in approximately 20% of vessels sampled being eliminated based on this criterion. The maximum diameter of individual vessels was averaged for each measuring frame. Finally, vessel number (vessels per mm^2) was calculated by allowing the image analysis system to count all vessel profiles in the measuring frame.

For comparisons between uninjured control, injured non-grafted, and injured grafted animals sample regions were taken from four similar spinal levels (see Fig. 4B). The first two, at the rostral and caudal interfaces, were selected regions of host gray matter (100 μm^2) that directly abutted graft tissue. In control-injured animals, similar regions were selected from surviving gray matter adjacent to the injury syrinx that was devoid of inflammatory cells or cystic cavitation. Three-dimensional reconstruction of the chronic contusion injury site has demonstrated that transplants fill the lesion syrinx but do not cause gross displacement or destruction of host gray matter (Stokes and Reier, 1992). As a result, these measurements represent a quantitative assessment of anatomically similar gray matter regions between animals with and without grafts. Two other measurements in each animal were made in the host gray matter 3 mm rostral and caudal to the point of impact (T_8). At these levels, graft tissue was sometimes apparent in white matter (e.g., dorsal columns or lateral funiculus), but did not directly interface with host gray matter. A minimum of five measurements were made at each described region in all animals. In animals receiving a transplant, additional measurements were made within the grafted tissue itself.

Cytochrome oxidase and peroxidase histochemistry

A modification of the technique of Hevner and Wong-Riley (1990) was used to stain for COx. For tissue used in quantitative analysis, spinal cords were removed rapidly under anesthesia and frozen in isopentane chilled to −50°C. For the purpose of histology alone, animals were perfused with warm (0.9%) saline followed by a solution of cold paraformaldehyde (2.5%) and glutaraldehyde (1.5%) in 0.1M phosphate (pH 7.4). Spinal cords were removed, rinsed with buffer, and cryo-protected in increasing concentrations of sucrose (10–30%) in 0.1M phosphate buffer over 1–2 days.

For COx quantitation, only fresh frozen sections (20 μm) were used. Until use they were stored at −70°C and subsequently reacted with diaminobenzidine (DAB). Such an approach was used in order to reduce variability in reaction rates induced by tissue fixation and to allow the short incubation times necessary to insure the linear progression of the DAB reduction process (van Raamsdonk et al., 1987; Kugler et al., 1988). All experimental and control sections for quantitation were reacted at the same time to avoid artifactual staining intensity differences that could evolve from slight variations in protocol. Tissue sections were immersed in a DAB substrate mixture (50 mg DAB, 100 ml of a 0.1M phosphate buffer at pH 7.35–7.40, 30 mg Cytochrome C, type III, and 4 g sucrose) at room temperature. Sections were allowed to react for 25 minutes when a light-dark brown reaction product appeared. For assessment of endogenous peroxidase activity, Cytochrome C was excluded from the incubation media and replaced with 0.02% hydrogen peroxide. Control sections were simultaneously arrested metabolically with 0.1M potassium ferricyanide to control for background staining. The sections were then rinsed in three changes of 0.1M phosphate buffer, air dried, and coverslipped. Alternate slides were also counterstained with cresyl violet.

Quantitation of cytochrome oxidase

Spinal cord tissues reacted for COx were quantitated using computer-assisted image analysis. Individual fields were selected (3/section; 10 μm^2) from tissue sections using an MCID M4 Image Analysis System (Imaging Research) under brightfield illumination. A digitized image was captured and regions of interest were examined in graft and host gray matter, being careful to avoid large relative populations of vascular elements. An optical density value was established for each 10 μm^2 region and all sections were analyzed under identical lighting conditions. The optical densities were averaged for each frame and combined for all sections prepared at the same time. Sample regions were taken from the same spinal areas described for the analysis of vascular surface fraction (see Fig. 4B).

Statistics

All data within a group (e.g., COx or vascularization) were statistically compared using a one-way ANOVA. All groups deemed significantly ($P < 0.05$) different were further analyzed with a Tukey-Kramer Multiple Comparisons post-hoc analysis.

RESULTS

Quantitative estimation of vascularization

In lesioned animals, the epicenter contained few vascular elements and was characterized by cystic cavities, phagocytic cells, and occasional, spared neuropil (Fig. 1A). The cysts were typically walled off by a continuous, thin layer of astrocytes (Fig. 1B). At 3 months post-injury, white matter 3 mm rostral to the injury epicenter showed evidence of persisting spongiform degeneration and increased numbers of large diameter blood vessels (Fig. 1C). However, at this time period, neither ventral or dorsal gray regions showed increased vascularization (Fig. 1D).

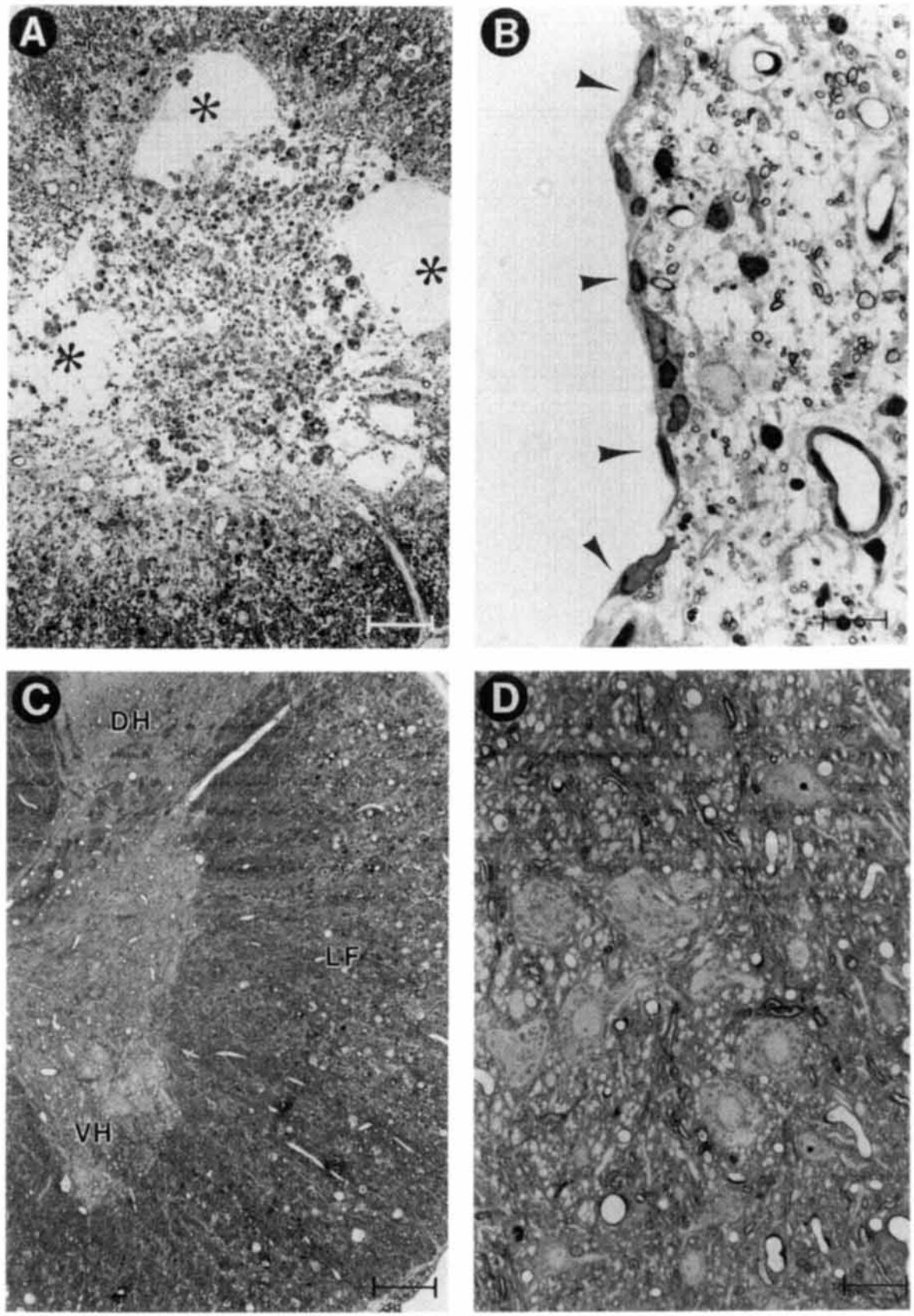

Fig. 1. Toluidine blue stained, 2-μm plastic cross-sections of rat spinal cord 3 months post-contusion injury. **A:** Injury epicenter showing cystic cavitation (asterisks), degenerating white matter, and phagocytes (scale bar = 100 μm). **B:** Higher magnification of A showing degeneration, gliotic barrier (arrowhead), and paucity of vascular elements (scale bar = 25 μm). **C:** Spinal cord region 3 mm caudal to injury epicenter (DH = dorsal horn, VH = ventral horn). Note degenerating white matter in lateral funiculus (LF) (scale bar = 100 μm). **D:** High magnification of C showing degenerating ventral gray matter. Note low number and size of vascular profiles (scale bar = 25 μm).

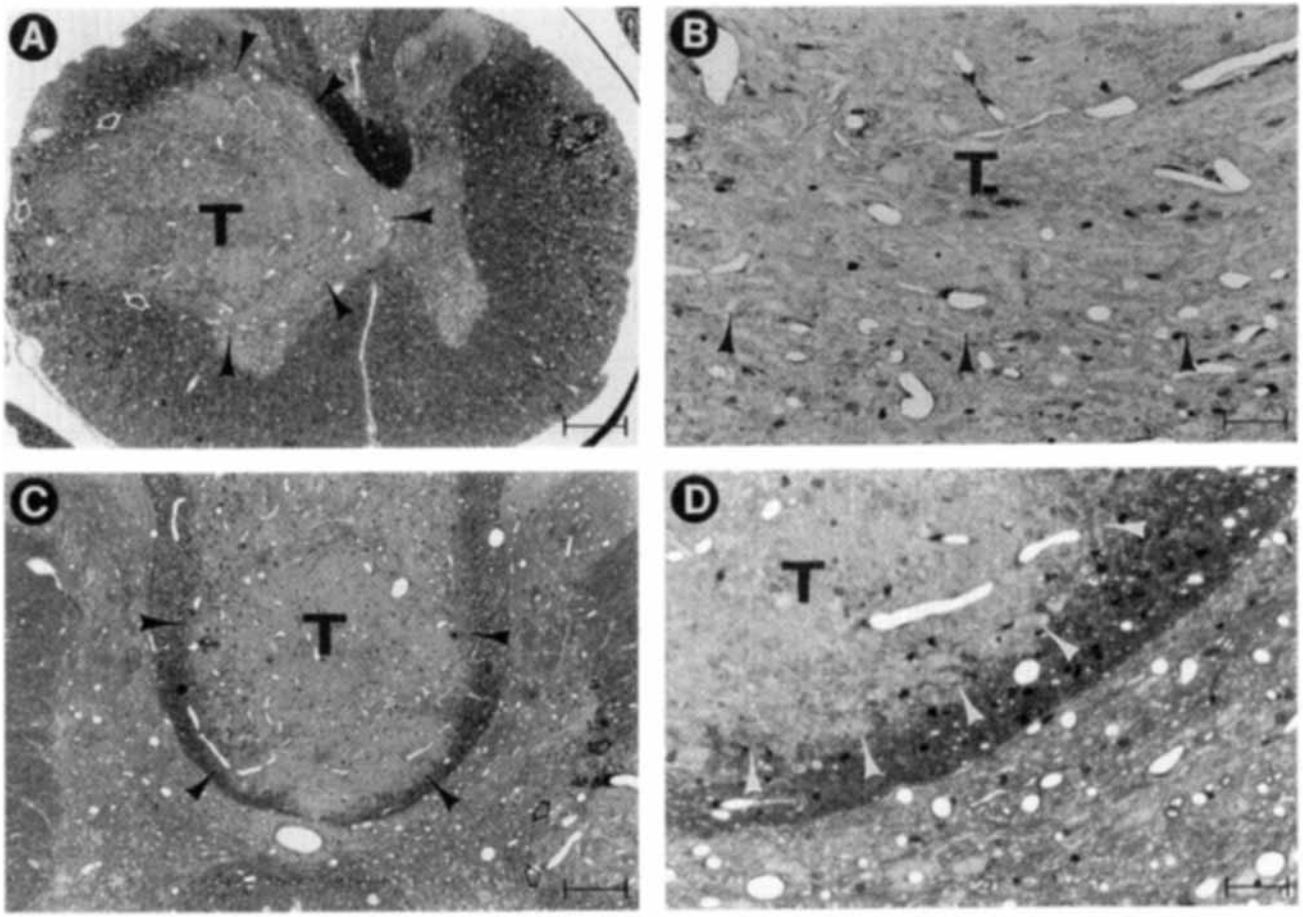

Fig. 2. Thin plastic cross-sections of rat spinal cord 3 months after an intraspinal transplant. **A:** Central region of fetal transplant (T). Note that the graft fills the entire lesion syrinx and interfaces with host gray matter (arrowheads) and host white matter (open arrows) (scale bar = 100 μm). **B:** High magnification of A showing region of transplant (T) apposed with the host intermediate gray matter. Note integration at the interface (arrowheads) between transplant (T) and host gray matter (scale bar = 25 μm). **C:** Section 3 mm rostral to injury epicenter. The transplant (T) dissects the dorsal funiculus and interfaces with host white matter (arrowheads). A small portion of the graft lies adjacent to the intermediate gray matter (open arrows) (scale bar = 100 μm). **D:** Higher magnification of C. Note well apposed interface (arrowheads) between transplant (T) and the dorsal funiculus (scale bar = 25 μm).

All transplants exhibited similar degrees of integration and morphology as previously described (Stokes and Reier, 1992). In general, the grafts contained a dense neuropil with well-differentiated neurons. These regions were interspersed with large bundles of fasiculated white matter. Regions consisting of small, tightly packed cells also were seen that resembled the substantia gelatinosa-like complexes described by Jakeman et al. (1989). In general, the grafts filled what otherwise would have been large central cavitations at the lesion epicenter and beyond. Confluent interfaces, devoid of extensive astroglial scarring, also were established (as described in Stokes and Reier, 1992) between regions of host gray matter and the graft neuropil at the rostral and caudal levels of the lesion zone (Fig. 2A,B). In addition, tapered aspects of the injury-induced syrinx, rostral to the epicenter, were completely filled by the graft tissue (Fig. 2C). Graft gray matter also had well integrated interfaces with host white matter despite the presence in some instances of degenerating myelin profiles (Fig. 2D).

Regions of the graft (Fig. 3B) with minimal white matter had many of the mature, small diameter capillaries characteristic of normal adult gray matter (Fig. 3A). The same was true of graft regions consisting of abundant white matter (Fig. 3C,D); however, grafts also contained many larger blood vessels (8–20 μm in diameter). Unlike arterioles, these vessels did not contain smooth muscle cells.

Spinal gray matter in uninjured control rats contained mostly small diameter vessels and had a consistant vascular cross-sectional percentage (ventral and dorsal gray) in all thoracic regions analyzed (Table 1 and Fig. 4A). Injury control animals did not show a significant reduction in vascularity (e.g., surface fraction) in spinal gray matter at the rostral or caudal injury margins of the lesions (Fig. 4A). The blood vessel surface fraction of transplanted tissue, however, was significantly greater than that of host spinal gray matter or the lesion epicenter (essentially avascular) of injured, non-grafted animals (Fig. 4A). In addition, a significant increase was detected in vascularity of interface host gray matter from grafted animals in comparison to gray matter from the same spinal level in injured, non-grafted animals or uninjured controls. Graft vessel number was significantly reduced in comparison to either normal or injured spinal gray matter at all spinal levels examined (Table 1). However, average vessel diameter was signifi-

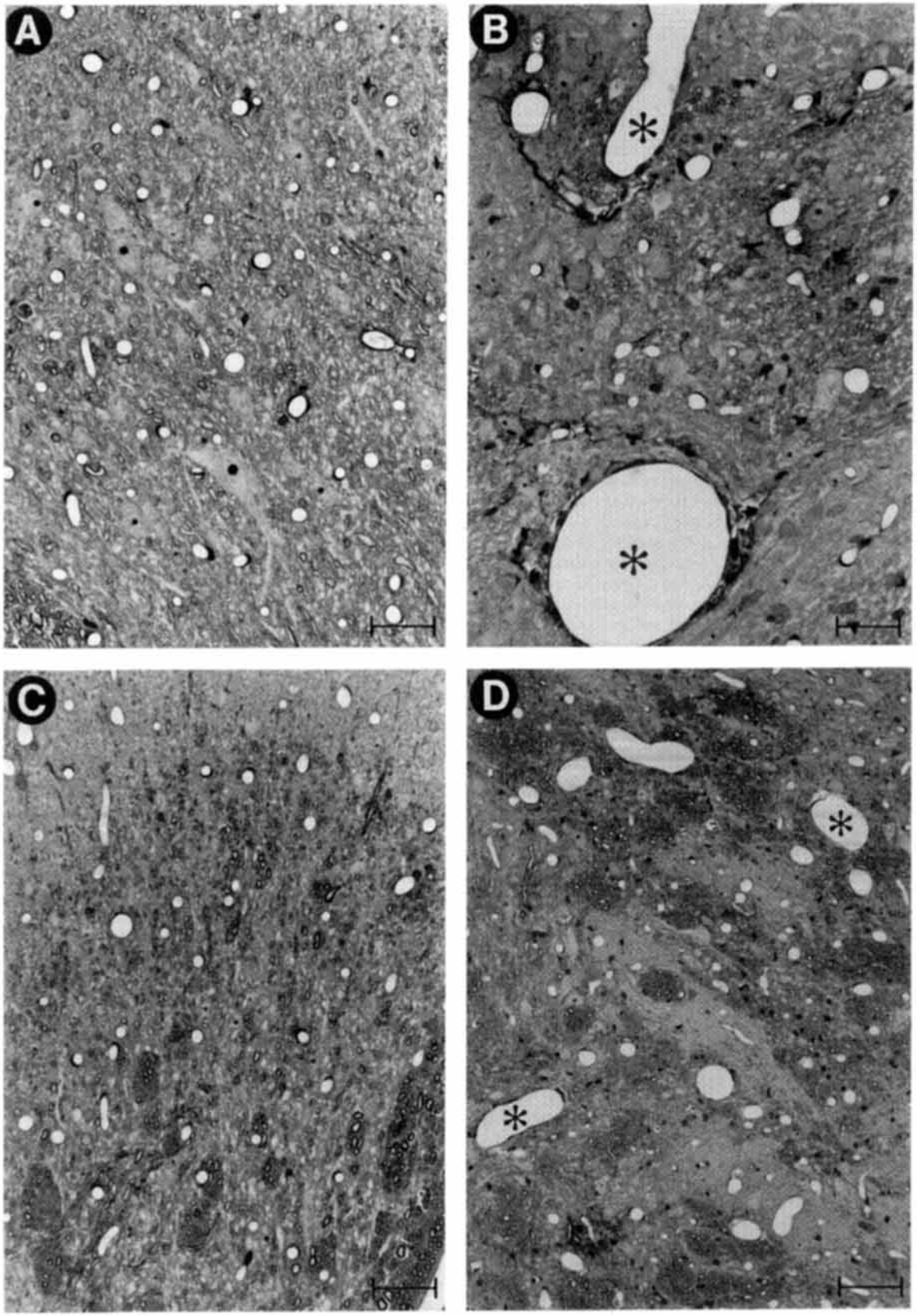

Fig. 3. Plastic cross-sections of normal rat intermediate gray (**A,** scale bar = 25 μm) and normal dorsal horn (**C,** scale bar = 50 μm) showing vascularization of gray matter containing relatively low or high amounts of interspersed myelinated axons, respectively. Photomicrographs **B** (scale bar = 25 μm) and **D** (scale bar = 50 μm) show graft regions containing low or high amounts of white matter, respectively. Note the relatively high number and small size of blood vessels dispersed through the normal intermediate and dorsal horn gray matter from non-injured animals. In the transplanted animal, vessels are irregularly oriented, and often contain very large penetrating vascular profiles (asterisks). Note the healthy appearance, however, and lack of perivascular cuffing in graft vessels.

TABLE 1. Results of Blood Vessel Quantitation[1]

	Average vessel number/mm²			Average vessel diameter (μm)		
	3 mm rostral/caudal	Rostral/caudal interface	Epicenter	3 mm rostral/caudal	Rostral/caudal interface	Epicenter
Uninjured control	91 ± 5	85 ± 7	87 ± 9	5.17 ± 0.63	5.25 ± 0.64	5.23 ± 0.75
Injured non-grafted	85 ± 9	93 ± 8	0	5.31 ± 0.54	5.15 ± 0.85	NA
Injured + graft	91 ± 8	68 ± 7*	64 ± 8*	5.23 ± 0.75	6.48 ± 0.74*	6.62 ± 0.83*

[1]Values are averaged for rostral and caudal regions and are reported as means ± standard deviation.
*$P < 0.05$ when compared to uninjured control at the same spinal level.

cantly increased in graft and host interface gray matter when compared to gray matter at the same spinal level in either injured, non-grafted, or uninjured controls. Therefore, increased vessel diameter and not vessel number contributes to the large vascular surface fraction measured in these regions.

Cytochrome oxidase and peroxidase histochemistry

Histochemical reaction of injured spinal tissue for COx revealed normal staining at spinal segments several millimeters (e.g., spinal T_5) from the cystic cavity at the lesion epicenter (Fig. 5A,B). For example, normal tissue reacted simultaneously with injured gray matter, and exhibited similar staining intensity and distribution patterns of dark, medium, and lightly reacting neurons. Cytochrome oxidase staining was confined primarily to neuronal somata and processes in both control and lesioned animals (Fig. 5C). The injury epicenter contained little gray matter capable of carrying on oxidative activity as reflected by the low level of diaminobenzidine (DAB) reaction product found within and adjacent to the contusion site (Fig. 5D). Surviving gray matter near the injury site had a decreased level of reaction product except for some large neurons (especially caudal to the lesion) that exhibited more intense staining than seen in spinal gray matter more distant from the injury site (Fig. 5D).

Cytochrome oxidase histochemistry demonstrated an increased metabolic potential in grafted fetal tissue. At the injury epicenter, a heterogeneous neuronal expression of DAB was seen (Fig. 6A) in donor tissue. Examination of semi-thin plastic sections suggested that lightly reacting regions corresponded to fasiculated white matter bundles coursing through the graft. At the host/graft interface large numbers of darkly reactive cells can be seen (Fig. 6B). It also was noted that host gray matter reacts less intensely than the adjacent transplant.

A quantitative evaluation of cytochrome oxidase expression was conducted. In lesion-only controls, cytochrome oxidase expression in host gray matter adjacent to the lesion syrinx (Fig. 4B) was reduced relative to gray matter 3 mm away from the injury epicenter or gray matter from uninjured controls (Fig. 4A). Conversely, host tissue at the host/graft interface had increased COx expression above injury control gray matter at a similar spinal level (Fig. 4A). When regions of graft gray matter and host gray matter were compared densitometrically, graft cytochrome oxidase expression was significantly higher than either neighboring or distant spinal gray matter (Fig. 4A). Finally, alterations in tissue COx appeared to roughly correlate with changes in vascular surface fraction.

Peroxidase histochemistry alone showed negligible endogenous tissue peroxidase activity. Injured gray matter and transplanted tissue showed low background levels of DAB when the reaction occurred under conditions favoring endogenous peroxidase (Fig. 6C). Regions exhibiting notable peroxidase reaction product were often found at the injury epicenter of lesion-only controls where small necrotic regions contained mostly phagocytic cells (Fig. 6D).

DISCUSSION

The goal of the present study was to describe concomitant vascular characteristics in mature intraspinal fetal transplants using quantitative analysis of vessel profiles and histochemical assessment of metabolism. In addition, the relationship between these parameters in spinal gray matter after contusion injury alone or in the presence of a fetal graft was studied. The results demonstrate that intraspinal grafts of fetal CNS tissue are capable of reaching an enhanced metabolic status and promoting a strong vascular proliferative response in the contused spinal cord. In addition, the presence of fetal grafts is associated with enhanced vascularization and COx expression at the host/graft interface. The ability of a well-established transplant to influence host neural tissue metabolism may be an important aspect of the functional alterations seen in other transplantation studies (Gage and Bjorklund, 1986; Stokes and Reier, 1991).

Graft angiogenesis

This work is part of a series of studies from this laboratory that seek to explore correlative expression of a variety of anatomical and functional indicators of successful intraspinal graft procedures (Stokes and Reier, 1990a, 1991, 1992). In the present experiments, image analysis was used to determine the vascularization of fetal grafts 3 months after placement in the contused rat spinal cord. In order to make reproducible, automated comparisons of host and graft blood vessels (representing anisotropic and isotropic structures, respectively), we utilized appropriate stereological methods (Weibel, 1979) and a computerized image analysis system for accuracy. Using these techniques, the volume of vascular elements (e.g., blood vessel surface fraction) was found to be significantly larger than adjacent host or normal spinal gray matter. In addition, graft blood vessels were larger in diameter and exhibited a more tortuous orientation than normal adult spinal vasculature. The random order of intraspinal transplant vessels agrees with earlier qualitative descriptions of vessel angioarchitecture in other suspension transplantation paradigms (Broadwell et al., 1991). Clearly the normal developmental queues that guide vascular growth in the embryonic spinal cord are altered in the post-contusion environment. In this regard, this altered graft vasculature may provide an unusual substratum for astrocytes and neurons to migrate or extend processes into the transplant (Krum and Rosenstein, 1988). In this manner, angiogenesis may not only provide metabolic fuel for early graft survival but tropic factor support as well which may influence patterns of

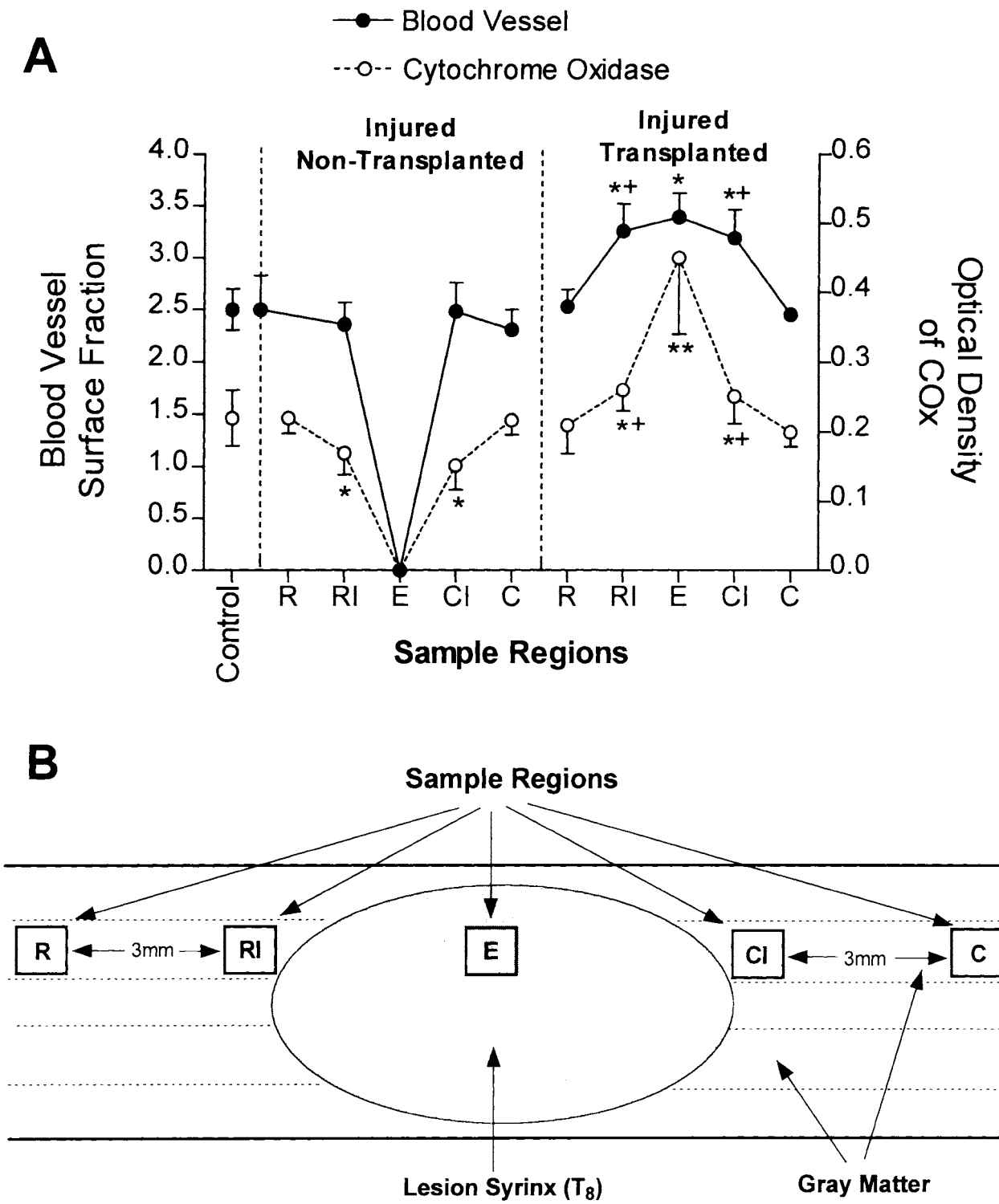

Fig. 4. Quantitation of vascularization and cytochrome oxidase. **A:** Injured control animals (100 days post-injury) do not show any significant variation in blood vessel surface fraction (dark circles) in spinal gray matter 3 mm rostral or caudal to the injury epicenter, or at the rostral or caudal interface. The injury epicenter is devoid of vascular elements at this time. Animals receiving a graft have increased vascular surface fractions (dark circles) both in host tissue at the host graft interface and in graft tissue within the lesion epicenter when compared to similar spinal levels in injured, non-grafted animals and uninjured controls. The optical density of COx (open circles) is significantly reduced in both gray matter at the rostral and caudal gray matter zones adjacent to the lesion syrinx and in the injury epicenter itself. Alternately, COx is elevated in host gray matter at the interface between graft and host and within the graft itself when compared to injured, non-grafted animals and uninjured controls. **B:** Diagrammatic representation of sampling regions for the data presented in A. R and C = 3 mm rostral or caudal, respectively, to the interface measurements; RI and CI = gray matter at the interface (rostral and caudal, respectively) between spinal gray matter and either the lesion syrinx for injured, non-transplanted or the graft in injured, transplanted animals; E = measurements at the epicenter (T_8) which represents the syrinx in injured, non-transplanted animals and graft tissue in injured, transplanted animals. Values are presented as means ± standard deviation. $^*P < 0.05$ compared to control; $^{**}P < 0.01$ compared to control; $\dagger P < 0.01$ compared to injured, non-transplanted gray matter of the same spinal level.

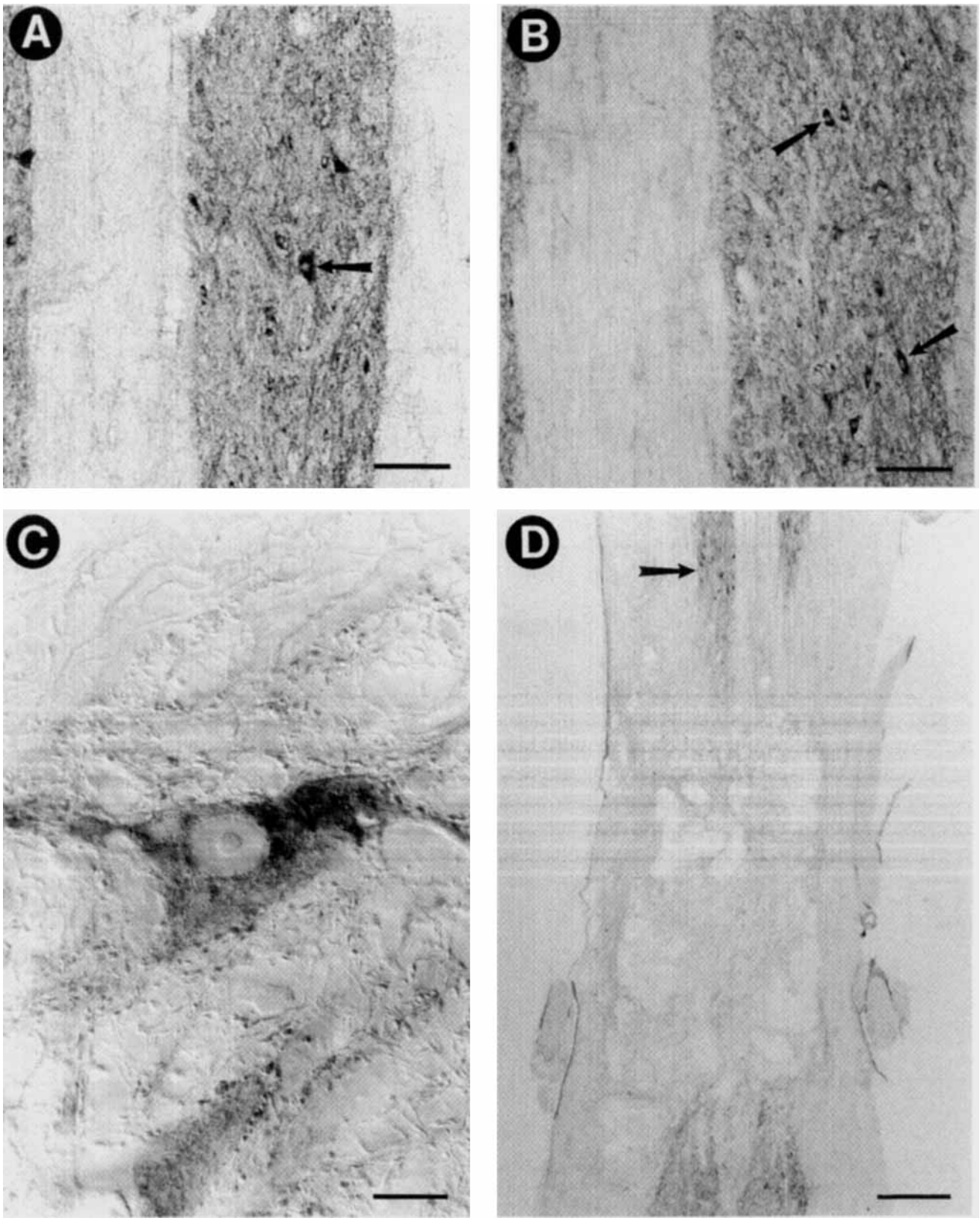

Fig. 5. Cytochrome oxidase reaction of spinal cord sections. **A:** Horizontal section through the normal dorsal horns. Many dorsal horn neurons and their processes are darkly reactive (arrow) (scale bar = 50 μm). **B:** Dorsal horns 3 mm rostral to the injury epicenter. Note the localization of the reaction product within the dorsal gray matter. Dorsal horn neurons (arrows) are clearly seen (scale bar = 50 μm). **C:** High magnification of a ventral horn neuron and processes. Note that the reaction product is localized primarily within the cell soma (excluding the nucleus) and neuronal processes. The staining intensity varies between neurons (scale bar = 10 μm). **D:** Horizontal section through the injury epicenter. Note the lack of staining within the cavity and reduced staining in the adjacent gray matter (arrow) (scale bar = 400 μm).

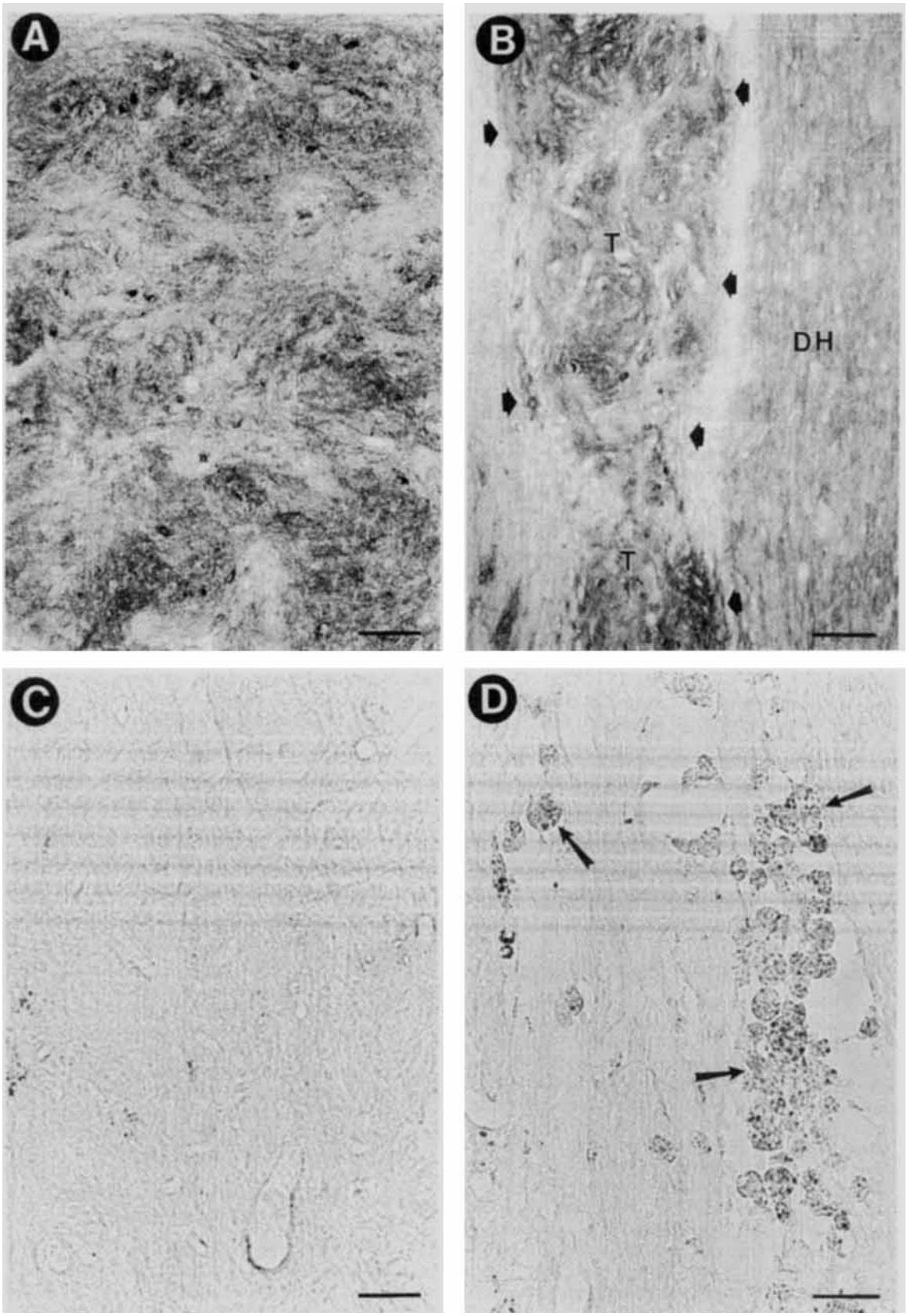

Fig. 6. Photomicrographs of cytochrome oxidase (A,B) and peroxidase (C,D) histochemistry (scale bars = 25 μm). **A:** Horizontal section through center of graft. Note high number of darkly reactive cells and neuropil. Light, non-reacting regions result from large bundles of myelinated fibers. **B:** In this horizontal section the interface between the transplant (T) and the host dorsal horn (DH) can be seen (arrowheads). Note darkly reactive neurons at the interface. **C:** Horizontal section through center of graft showing only background peroxidase staining at 3 months post-transplantation. **D:** Horizontal section through injury epicenter (100 days post-injury). Note that peroxidase reaction product is localized within remaining phagocytes (arrows).

host/graft connectivity. Replacement of structural components of the vascular system in and around transplants, however, does not necessarily imply the existence of a tight coupling between such elements and local metabolism as occurs in the normal CNS (Gross et al., 1987; Black et al., 1991).

The current data suggest that the size of the graft vascular bed is associated with high graft metabolic capacity. Although the precise regulatory factors are unknown, there are many regulatory signals that initiate and maintain this process in the normal CNS. These include tissue O_2 level (P_tO_2), adenosine (Meininger et al., 1988), and lactate (Jensen et al., 1986). Of these, tissue oxygen levels probably play a key role in the early vascularization of intraspinal transplants. It has been previously demonstrated that fetal grafts are able to establish a low P_t O_2 (<20 mm Hg, torr) beginning 7 days after transplantation, and lasting for up to 3 months (Stokes and Reier, 1990a, 1991). Since high oxygen tensions inhibit endothelial proliferation, it may be essential for a fetal CNS graft to alter the abnormally high P_t O_2 which appears after spinal cord injury (Stahl and Harris, 1986; Stokes and Reier, 1991). In the normal CNS, low oxygen tensions can stimulate endothelial cells to migrate directly (Meininger et al., 1988) or proliferate under the influence of angiogenic factors released from macrophages (Knighton et al., 1983). Early angiogenic processes also have been associated with macrophage clusters in developing grafts (Zhou et al., 1986). These metabolic factors influencing angiogenic responses probably also play a role in alterations we have described in adjacent host tissue.

Enhanced vascularity that occurs rostral or caudal to the host/graft interface is clearly in excess of that which appears as a result of injury alone. This suggests that the increase in vascular surface fraction in this region is likely a result of graft-induced alterations in the post-traumatic extracellular milieu. In this regard, interface regions also exhibit low oxygen tensions in the presence of an intraspinal transplant (Stokes and Reier, 1991). Taken together with results in other lesion models in our laboratory, these data suggest that grafts are highly metabolically active and are capable of inducing such changes in the nearby host neuropil.

Metabolism in intraspinal fetal transplants

The present data indicate that embryonic fetal spinal cord tissue, transplanted at a subchronic time period after spinal cord contusion injury (e.g., 10 days), results in increased COx reactivity. We assume that graft cells are highly physiologically active because the presence of this enzyme has been previously shown to correlate directly with synaptic activity of nerve cells (Wong-Riley et al., 1981; Chalmers et al., 1992; Goldberger et al., 1993). In both control and injured spinal cord tissue, the DAB reaction product was localized within neurons. Only very limited staining was evident within either astrocytes or oligodendroglia. Increased COx reactivity in conjunction with high levels of endogenous peroxidase have been described in the spinal cord 2 weeks after contusion injury (Noble et al., 1990). In the present experiments, however, spinal cord tissue 3 months after contusion injury had reduced COx expression both within the lesion cavity and in neighboring spinal gray matter. In addition, reaction of injured spinal tissue for endogenous peroxidase alone revealed background levels with the exception of small clusters of macrophages within the lesion syrinx. The results of these histochemical tests indicate that increased COx density within transplanted tissue and the neighboring host neuropil is due to the presence of a well-developed intraspinal fetal transplant.

In the present model, where graft development is allowed to proceed for 3 months, donor tissue metabolism was at least qualitatively matched to an appropriate blood supply. This is in contrast to other grafting paradigms where an uncoupling of metabolic need and vascular volume has been described (Sharkey et al., 1991). In addition, the increase in host gray matter expression of COx at the host/graft interface indicates that such patterns of metabolism are transferred to adjacent tissue in sharp contrast to post-contusion gray matter in animals not receiving a spinal graft. Furthermore, since these alterations occur only at short distances from the interface, they roughly correlate with patterns of short range host-graft axonal elongation in similar models (Jakeman and Reier, 1991). Because grafted neurons also have increased levels of COx over normal spinal gray matter, it may indicate that differentiation of this tissue has been influenced by reciprocal or host-derived innervation.

Clearly these patterns do not represent the normal synaptic interactions found in the adult spinal cord; however, increased graft cellular metabolism may reflect functional host/graft connectivity. For example, focal regions of increased COx expression in fetal tectal grafts has been shown to correlate directly with patterns of functional innervation from the host (Dyson et al., 1988). Neurotransmitter expression within the graft alone, however, was not a good predictor of graft metabolism. Instead, graft regions that lacked direct host innervation expressed low levels of COx. A similar relationship has been described between metabolism (assessed by 2-deoxyglucose autoradiography) and functional host/graft innervation in septal, cortical, and substantia nigra transplants (Schmidt et al., 1982; Kelly et al., 1985; Ebner et al., 1989). These data indicate that increased levels of COx in fetal spinal grafts may be indicative of a high level of synaptic activity. Much of this signal may be derived from host fibers. In this regard sensory fibers, especially CGRP immunoreactive fibers from the dorsal root, have been shown to have a robust regenerative capacity after spinal lesioning and transplantation (Tessler et al., 1988a,b; Houle and Reier, 1989). Given that intraspinal transplants are capable of differentiating into sensory regions that resemble the normal spinal dorsal horn (Jakeman et al., 1989), increased cytochrome oxidase expression in grafted neurons may indicate considerable functional sensory innervation from the host. Future experiments combining immunohistochemical or retrograde tracer analysis with the metabolic studies presented here are needed to determine if such synaptic integration contributes to the metabolic rate of grafted cells.

Regulators of angiogenesis and graft metabolism

Variables which may influence vascularization and graft metabolism include the 1) immunological status of the host, 2) age and/or region of origin of the fetal tissue, 3) physiological status of the transplant site, 4) procedure by which the tissue was prepared for transplantation, and the 5) region of the CNS in which cells are to be grafted. While immunological rejection is one of the largest obstacles of fetal grafting in general, some fetal allografts can survive

long periods when placed into the CNS. In our model, transplants show no overt signs of rejection. Vascular profiles typically lack perivascular cuffing and immune infiltrates are uncommon at 3 months post-transplantation. It is generally agreed that transplants are capable of increased expression of MHC antigens and lymphocyte infiltration (Lawrence et al., 1990; Sloan et al., 1991; Broadwell et al., 1994); however, the conditions under which allografts reject is uncertain. In addition, grafting into a previously lesioned region of the CNS can lead to immune cell infiltration without cell destruction (Duan et al., 1993). This may be due to a form of immune deviation following CNS injury (Wilbanks and Streilein, 1992; Horner et al., 1993). Despite the lack of an aggressive rejection response, it should be noted that immune cells may play a role in graft development. For example, factors released by immune cells (e.g., IL-1, TGF-β) could contribute to vascularization and/or cellular metabolism at focal sites within a graft. The presence of immune-stimulated vascular growth within the injured host after spinal cord injury may also contribute to graft survival and development (Blight, 1991). Such processes could also lead to an uncoupling between graft metabolism and angiogenesis and may possibly explain the disparity between these factors described in other transplant paradigms (Sharkey et al., 1991). In the present experiments, lack of dramatic immune infiltration and abnormal perivascular spaces (3 months post-transplantation) indicates that graft and host interface vascularization is not largely a result of such graft rejection phenomena.

Secondly, it is conceivable that manipulations associated with tissue preparation could cause temporary cell trauma that might affect a graft's ability to form synaptic connections, or respond to or release growth factors. Under such circumstances, low graft metabolism and/or vascularization would be expected to result in poor graft survival and integration. However, a variety of donor preparation procedures for transplantation into the CNS have been described that consistently lead to viable transplants (Schmidt et al., 1983; Bjorklund et al., 1983; Dunnett and Bjorklund, 1992). In our experience, grafts have good growth potential and routinely develop a healthy syncytium with the host spinal cord.

Finally, evidence exists from other transplantation models that graft location and condition of the transplantation site (e.g., whether undisturbed, post-traumatic, or undergoing degeneration) most likely influences many aspects of transplant maturation and metabolism. For example, when fetal grafts of the same origin are placed in different regions of the CNS, graft metabolism varies depending upon the graft site (Rosenstein, 1993). In addition, when the graft origin is varied but the transplantation site is held constant, similar metabolic rates are observed (Lu et al., 1991). With respect to the condition of the transplantation site, prior lesioning of the host can lead to increased host sprouting and innervation of transplanted cells (Needels et al., 1986; Nieto-Sampedro et al., 1987) with concomitant up-regulation of graft metabolism (Ebner et al., 1989). One factor that may play a role in this process is bFGF which can stimulate angiogenesis and neuronal differentiation (Risau et al., 1988; Sweetnam et al., 1991) and is also up-regulated after spinal cord injury (Koshinaga et al., 1993). Similar injury-induced factors may play an important role in our 10 day post-injury transplantation paradigms. Clearly the environment created by the contusion lesion contributes to the differentiation of the graft and its ability to influence host metabolism.

CONCLUSIONS

The present study suggests that a metabolic indicator, cytochrome oxidase, and vascular development are qualitatively associated in a matured intraspinal graft of fetal CNS tissue. Several mechanisms could account for the level of metabolic activity achieved in this transplant paradigm. However, results from other laboratories suggest that increased levels of cytochrome oxidase within the transplant may indicate functional graft/host integration. The ability of the graft to influence host metabolism at the host/graft interface suggests that the presence of this tissue alters the synaptic frequency of neighboring host cells. This indicates that the normal decrease in metabolic activity in host neurons near the lesion syrinx can be altered by the presence of fetal graft neurons. Since metabolic alterations are still present after 3 months, these results imply that a long lasting, functional alteration in the synaptic input to host interface neurons occurs after transplantation. In the future, an analysis of the source of fiber types that may be involved is needed combined with axotomy to alter the expression of cytochrome oxidase in the graft. In addition, a more quantitative, longitudinal approach to estimate host metabolism (e.g., glucose uptake) would be useful for making comparisons of the chemical nature of graft and host metabolism as it relates to other transplantation models.

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