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PC12 cells differentiate into chromaffin cell-like phenotype in coculture with adrenal medullary endothelial cells

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ABSTRACT **Previously we described specific *in vitro* interactions between PC12 cells, a cloned, catecholamine-secreting pheochromocytoma cell line derived from the rat adrenal medulla, and bovine adrenal medullary endothelial cells. We now demonstrate that these interactions induce the PC12 cells to acquire physical and biochemical characteristics reminiscent of chromaffin cells. Under coculture conditions involving direct cell–cell contact, the endothelial cells and the PC12 cells reduced their rates of proliferation; upon prolonged coculture PC12 cells clustered into nests of cells similar to the organization of chromaffin cells seen *in vivo*. Within 3 days in coculture with endothelial cells, but not with unrelated control cells, PC12 cells synthesized increased levels of [Met]enkephalin. In addition, PC12 cells, growing on confluent endothelial monolayers, failed to extend neurites in response to nerve growth factor. Neither medium conditioned by endothelial cells nor fixed endothelial cells could by themselves induce all of these different phenomena in the PC12 cells. These results suggest that under coculture conditions PC12 cells change their state of differentiation toward a chromaffin cell-like phenotype. The rapid, transient increase in the expression of the protooncogene c-*fos* suggests that the mechanism(s) inducing the change in the state of differentiation in PC12 cells in coculture with the endothelial cells may be distinct from that described for the differentiation of PC12 cells—e.g., by glucocorticoids. We propose that similar interactions between endothelial cells and chromaffin cell precursors may occur during embryonic development and that these interactions might be instrumental for the organ-specific differentiation of the adrenal medulla *in vivo*.**

The organ-specific differentiation of neural crest-derived cells, such as adrenal medullary chromaffin cells, greatly depends on cues present in the local environment (1, 2). PC12 cells, a catecholamine-secreting pheochromocytoma cell line cloned from the rat adrenal medulla (3), have been a useful model system to study the effects of such cues on neuronal differentiation (4). Thus, in the presence of nerve growth factor (NGF), PC12 cells will differentiate into sympathetic neurons (5). Alternately, a chromaffin cell-like phenotype can be obtained upon exposure of the PC12 cells to glucocorticoids (6, 7) or to sodium butyrate (8, 9) or by growing them on fibronectin (10, 11). In this context, a chromaffin cell-like phenotype has been defined by the following characteristics: PC12 cells remain rounded and appear bright under phase-contrast optics; the number and the size of the catecholamine storage granules increase; their contents of neuropeptides and their secretory response are elevated (8, 9).

The cellular sources for the various environmental stimuli responsible for the organ-specific differentiation of neural crest-derived cells remain unclear. We recently hypothesized that vascular endothelial cells may also be involved in regulating the pathway of neural crest cell differentiation (12). Indeed, Carmichael *et al.* (13) suggested that capillary endothelial cells might regulate the neonatal development of chromaffin cells in the opossum adrenal medulla.

We have approached this question by coculturing bovine adrenal medullary endothelial (BAME) cells (14) with PC12 cells and demonstrated that specific adhesive interactions occur between PC12 and the BAME cells that are mediated by proteinaceous factors residing in their respective cell surfaces (15). We now provide evidence suggesting that these interactions are accompanied by and/or lead to functional changes in both cells, which for the PC12 cells result in their differentiation into a chromaffin-like cell.

METHODS

Cell Cultures. PC12 cells and BAME cells were grown in Dulbecco's modified essential medium (DMEM) and in Eagle's modified essential medium, respectively, as described (15). For some of the coculture experiments, BAME cells were also adapted to grow in PC12 growth medium without affecting their growth rates or their morphology. Fibroblasts (NIH 3T3 cells) were grown in DMEM supplemented with 10% fetal bovine serum and 10 μg of gentamicin per ml. Urea cell surface extracts were prepared as described (15).

Cocultures. PC12 cells were seeded onto the contact-inhibited monolayer of BAME cells, onto NIH 3T3 controls, or onto untreated culture dishes and maintained in PC12 growth medium for up to 21 days (15). Viability of these cultures exceeded 95%, as assessed by trypan blue exclusion. The proliferation rates of PC12 and BAME cells were determined by counting nuclei in a Coulter Counter (15). In addition, we determined their individual rates of proliferation by differentially counting PC12 and BAME cells in a hemocytometer using a unique feature of the neutral red assay (16): PC12 cells avidly take up the vital stain neutral red, whereas the uptake of neutral red by BAME cells is limited. Following staining for 2 min with a saline solution containing 167 μg of neutral red per ml (from GIBCO), the PC12 cells appear dark red, whereas the BAME cells remain slightly pink and translucent. Counting the number of PC12 cells in the individual cell cultures, either in a Coulter Counter or manually,

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Abbreviations: BAME, bovine adrenal medullary endothelial; NGF, nerve growth factor.

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using the neutral red assay, yielded comparable results. The total rate of cell proliferation in the cultures was also assessed by measuring total protein contents over time and by pulse labeling the cells with [^{3}H]thymidine and determining thymidine incorporation: 1×10^5 endothelial cells were plated onto a 24-well plate, and after 24 hr PC12 cells (1×10^5) were added. Twenty-four hours later [^{3}H]thymidine (1 μCi per well; 1 Ci = 37 GBq) was added, the cells were harvested after another 24 hr, and the cell-associated radioactivity was determined. Total protein in the cultures was determined using the Bradford assay (from Bio-Rad) with bovine serum albumin as standard.

Conditioned Media. BAME or PC12 cells were grown for 72–96 hr in their own growth medium. The media were collected and centrifuged, and the supernatant was then considered conditioned medium. In some of the experiments detailed below, PC12 cells were gradually (within two passages) adapted to grow in BAME medium (and vice versa) without affecting their individual growth rates or the subsequent results. Conditioned medium was aliquoted and stored at −80°C until required.

Peptide Radioimmunoassay. Preconfluent cell cultures, grown for 1–3 days in six-well Costar plates, were washed with phosphate-buffered saline, and cell extracts were prepared with 0.5 ml of an acidic mixture containing 2 M acetic acid, 0.04 M HCl, and 0.2% 2-mercaptoethanol. [Met]enkephalin immunoreactivity was determined according to ref. 17 and is expressed as pmol/mg of protein.

Northern Blot Analysis. PC12 cells were grown for 30 min to 24 hr on confluent monolayers of cells or in control dishes. PC12 and BAME cells were separated after the stated times of coculture by gentle mechanical agitation, which dislodged the PC12 cells but left the BAME monolayer intact. Total RNA from both cell populations was separated on a formaldehyde/agarose gel and blotted onto nitrocellulose paper or a nylon membrane (GeneScreen). The murine genomic c-*fos* gene (18) was obtained through the American Type Culture Collection. An *Nco* I–*Sph* I fragment containing the fourth exon of the gene was subcloned into M13mp19. A ^{32}P-labeled single-stranded probe was prepared by primer extension reaction, and Northern blot hybridization was carried out according to ref. 19.

Microscopy. Samples for light and electron microscopic visualization were prepared as described (15). Cell cultures were grown in two-well plastic tissue culture/chamber slides (Miles), fixed in 3.7% formaldehyde, and viewed in a Nikon FX upright microscope using phase-contrast optics. Semithin sections and samples of the unfixed, live cocultures, grown in 35-mm plastic dishes, were photographed in a Zeiss IM 405 or a Nikon Diaphot TMD inverted microscope equipped with phase-contrast optics.

RESULTS

PC12 Cells Form Clusters on Endothelial Cell Monolayer. PC12 cells cultured in DMEM showed a low degree of cellular clumping: even at high densities only few, two-dimensional cell aggregates were observed (Fig. 1*A*). By contrast, PC12 cells readily aggregated and began to form clusters within 24 hr following seeding at low density (*ca.* 10^4 cells per cm^2) onto a monolayer of BAME cells (15). This clustered, three-dimensional organization of PC12 cells on top of the endothelial cell monolayer was particularly prominent when the cultures were maintained over the range of 1–3 wk (Fig. 1*B*). Semithick sections confirmed that these clusters were formed by an aggregation of individual PC12 cells and were 10–20

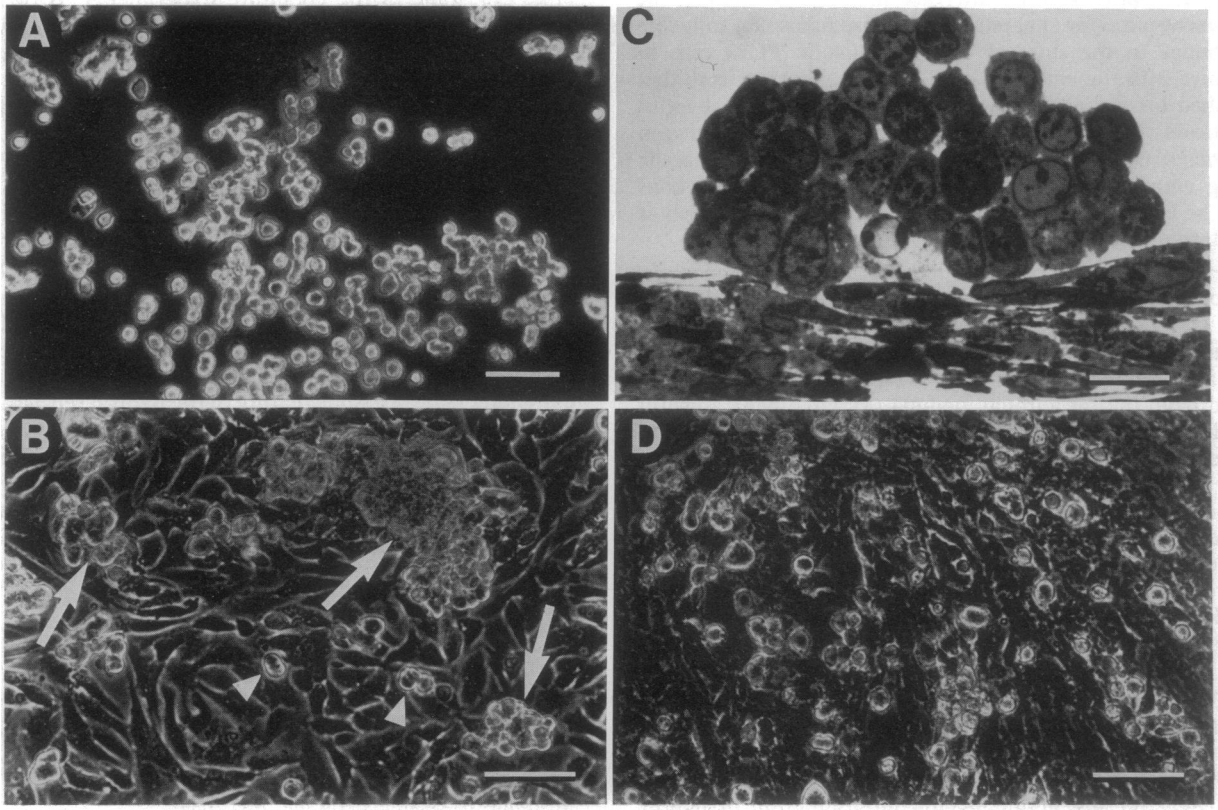

FIG. 1. Cocultures of PC12 cells with BAME cells. (*A*) PC12 cells seeded at 10^4 cells per cm^2 reach confluence within 5 days. (*B*) PC12 cells grown for 7 days on a confluent BAME cell monolayer. Individual PC12 cells remain rounded and phase-bright (arrowheads); frequently, PC12 cells were found in multicellular clusters of various sizes (arrows). (*C*) Semithick section of a characteristic, large PC12 cluster growing on BAME cells for 14 days. (*D*) PC12 cells grown for 10 days on a monolayer of NIH 3T3 fibroblasts. Note the absence of cluster formation of the PC12 cells. (Bars in *A*, *B*, and *D* = 100 μm; bar in *C* = 20 μm.)

cells thick (Fig. 1*C*). Such three-dimensional clusters could not be observed when we grew PC12 cells at the same initial density for 1 wk on confluent monolayer of NIH 3T3 fibroblasts (Fig. 1*D*) or on culture dishes coated with BAME-derived extracellular matrix (not shown).

Coculture Causes Reduced Proliferation of PC12 and Endothelial Cells. PC12 cells, when seeded at an initial density of 10^4 cells per well into six-well plates, reach confluence within 4–5 days. However, when seeded at the same density onto confluent monolayers of BAME cells, PC12 cells were found to survive without reaching confluency for up to 3 wk, suggesting a decrease in proliferation. We verified this notion by counting the total cell numbers in a Coulter Counter (Fig. 2*B*). This reduction in the rate of proliferation of the BAME cells and the PC12 cells was verified independently by pulse-labeling individual cultures of PC12 and BAME cells and their cocultures, respectively, with [^{3}H]thymidine for 24 hr. PC12 cells and BAME cells, grown alone, incorporated 4890 ± 193 cpm and 17,127 ± 1444 cpm of [^{3}H]thymidine, respectively. When both cells at the same respective densities were cocultured for 24 hr, thymidine incorporation was drastically reduced (6312 ± 302 cpm). Medium conditioned by the BAME cells also caused a reduced thymidine incorporation in PC12 cells alone by about 50%, although the actual number of viable cells did not vary significantly from the number of cells originally plated (not shown). In an independent set of experiments we also observed a decrease in the rate of protein synthesis in the cocultures, as compared to the individual cell cultures (Fig. 2*A*). It is noteworthy that in all of these experiments the viability of the cells (assayed by trypan blue exclusion) did not decrease. To further quantitate these observations, we measured the rates of proliferation of PC12 cells and BAME cells individually and in coculture using the neutral red assay. As seen in Fig. 2*C*, the rate of proliferation of both cells is significantly attenuated when the cells are cocultured in the same dish.

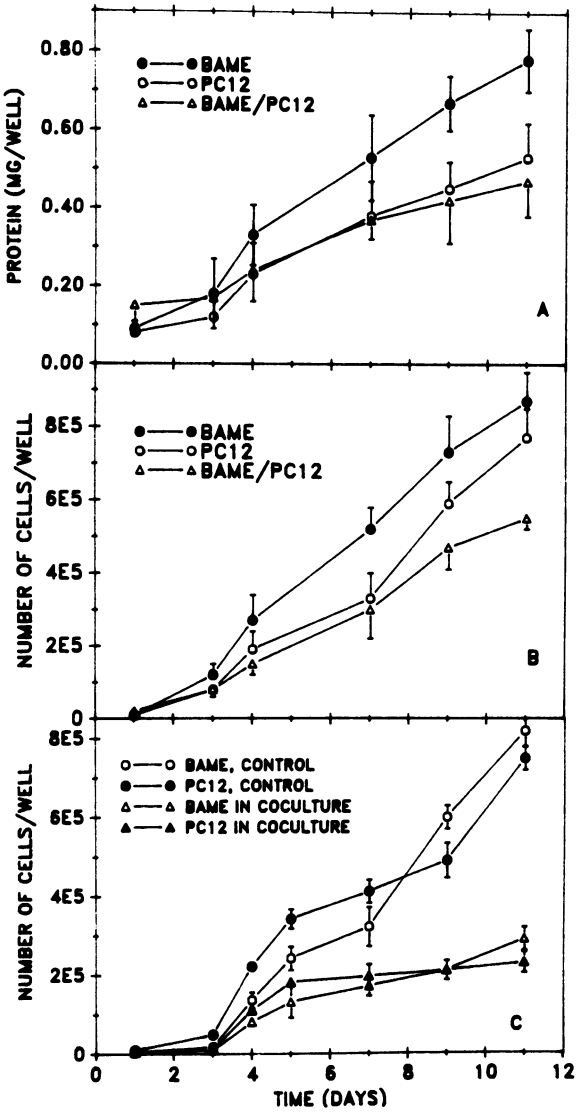

FIG. 2. Growth curves of PC12 cells and of BAME cells in coculture. (*A*) Total protein in the cell cultures adapted to grow in PC12 medium, determined by the Bradford assay. (*B*) Total cell numbers, as determined by counting nuclei (15), were determined in parallel experiments to those depicted in *A*. (*C*) Differential counting of the cells in a hemocytometer, using the neutral red assay. The data represent triplicate measurements in a typical experiment, which was repeated two more times with qualitatively similar results.

PC12 Cells Cocultured with BAME Cells Do Not Extend Neurites in Response to NGF Treatment. Within 24 hr after addition of 50 ng of NGF per ml, PC12 cells began to extend neurites (3); after prolonged continuous exposure of the cells to NGF (up to 14 days) a profound network formed (Fig. 3*A*). By contrast, PC12 cells from the same batch, seeded onto confluent monolayers of BAME cells and maintained in culture for up to 3 wk in the presence of up to 200 ng of NGF per ml, remained mostly rounded, did not lose their phase brightness, and could not be distinguished from cocultures not treated with NGF (Fig. 3*B*). Furthermore, neurite outgrowth, a typical response of PC12 cells to NGF, could not be demonstrated during up to 21 days in the continued presence of NGF, even when the cocultures were fed every other day.

Elevated [Met]Enkephalin Expression in PC12 Cells Cocultured with BAME Cells. Differentiation of PC12 cells toward

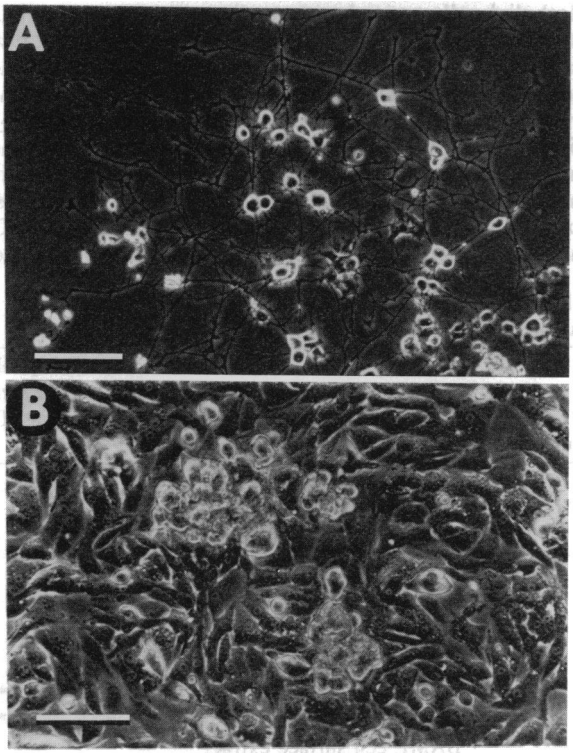

FIG. 3. Loss of NGF-induced neurite sprouting in PC12 cells cocultured with endothelial cells. (*A*) PC12 cells grown for 14 days in the presence of 100 ng of NGF per ml. (*B*) PC12 cells plated onto BAME monolayers did not extend neurites following treatment for 3 wk with 150 ng of NGF per ml. (Phase-contrast micrographs of live, unfixed specimen. Bar = 100 μm.)

chromaffin-like cells is accompanied by an increased level of [Met]enkephalin expression (8, 9). In line with these results we found only low levels of [Met]enkephalin immunoreactivity in PC12 cells cultured alone (Table 1). No [Met]enkephalin immunoreactivity was detected in BAME cells. We also did not find a significant difference in [Met]enkephalin expression between untreated control and NGF-treated PC12 cells within the time course studied. However after 3 days in coculture with BAME cells, [Met]enkephalin immunoreactivity in PC12 cells increased ≈2.5-fold (Table 1, group 6). Conditioned media or urea cell surface extract from either PC12 cells, BAME cells, or NIH 3T3 cells did not affect PC12 [Met]enkephalin immunoreactivity. Coculture of the cells for 3 days in the presence of 50 μg of BAME cell surface extract per ml also resulted in elevated [Met]enkephalin levels, albeit to a slightly lesser extent.

c-*fos* Expression in Cocultured Cells. c-*fos* expression is transiently elevated in PC12 cells treated with NGF or epidermal growth factor, although not when treated with glucocorticoids (20). To further elucidate the nature of PC12–BAME interactions, we assayed for changes in the level of c-*fos* mRNA expression by Northern blot analysis. Shown in Fig. 4 is the region of 2.2 kilobases, corresponding to the size of the c-*fos* message (20). In the particular experiment shown in Fig. 4, RNA was harvested after 45 min under the various experimental conditions. The level of c-*fos* mRNA expression in untreated PC12 cells is shown in Fig. 4, lane 1. As positive controls we measured c-*fos* expression in PC12 cells treated with 100 ng of NGF per ml (Fig. 4, lane 8) and in BAME cells treated with 100 ng of acidic fibroblast growth factor per ml, respectively (ref. 21; data not shown). c-*fos* mRNA was found to be transiently elevated under the following conditions: (*i*) PC12 cells cocultured with the BAME cells (Fig. 4, lane 6); (*ii*) PC12 cells treated with medium conditioned by BAME cells (Fig. 4, lane 2); and (*iii*) endothelial cells treated with medium conditioned by PC12 cells (not shown). In all of these cases c-*fos* expression was found to increase transiently, peaking (≈10-fold over background) after ≈30–45 min (data not shown), consistent with previous observations (22). Only minimal activation of c-*fos* (≈50–100% above background) was observed in three independent experiments in PC12 cells treated with medium conditioned by NIH 3T3 cells (Fig. 4, lane 3). No changes in c-*fos* levels were observed in PC12 cells treated with PC12 conditioned medium (Fig. 4, lane 4), in endothelial cells treated with medium conditioned by endothelial cells (not shown), or in PC12 cells treated with BAME cell surface extracts (Fig. 4, lane 5).

Table 1. [Met]enkephalin immunoreactivity in PC12 cells cocultured with BAME cells

Group	Experimental conditions	[Met]enkephalin immunoreactivity, pmol/mg of protein
1	PC12 cells (control)	0.28 ± 0.08
2	+ 50 μg of PC12 cell surface extract per ml	0.34 ± 0.06
3	+ 50 μg of BAME cell surface extract per ml	0.23 ± 0.02
4	+ 100 μg of NIH 3T3 cell surface extract per ml	0.21 ± 0.01
5	+ BAME conditioned medium	0.21 ± 0.05
6	+ BAME cells, cocultured	0.69 ± 0.19*
7	+ BAME cells + 50 μg of BAME cell surface extract per ml	0.55 ± 0.06*

[Met]enkephalin immunoreactivity was measured in PC12 cells cultured for 3 days under the various conditions. Data represent the mean ± SD of triplicate determinations.
*Data are statistically significant ($P < 0.001$) from the control experiments.

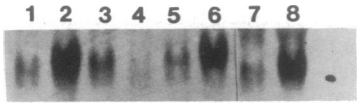

FIG. 4. c-*fos* expression in PC12 and in endothelial cells. Northern blot hybridization of total cellular RNA. Lane 1, PC12 cells grown on plastic (control); lane 2, PC12 cells plus BAME conditioned medium; lane 3, PC12 cells plus NIH 3T3 conditioned medium; lane 4, PC12 cells plus 100 μg of PC12 cell surface extract per ml; lane 5, PC12 cells plus 100 μg of BAME cell surface extract per ml; lane 6, PC12 cells plus BAME cells (cocultures); lane 7, BAME cells grown alone (control); lane 8, PC12 cells plus 100 ng of NGF per ml. All experiments were performed with PC12 cells adapted to grow in BAME cell medium. RNA was harvested after 45 min of coculture or incubation. Qualitatively similar results were obtained in two independent sets of experiments.

DISCUSSION

PC12 cells retain a key feature of neural crest-derived chromaffin cell percursor cells: depending on environmental stimuli, they will either differentiate toward sympathetic neurons or express a phenotype characteristic for small intensively fluorescent cells or chromaffin cells (4–10). Since migration of neural crest-derived cells often occurs along a preformed vascular bed or concomitant with the development of the vasculature (23), it is plausible to assume that some of these environmental stimuli—either humoral, such as heparin binding growth factor or related compounds (24), or contained in the extracellular matrix—might also originate from the vascular endothelial cells (25). For example, humoral signals derived from pulmonary endothelial cells might influence fetal lung development and differentiation (26). During early stages of development prior to the establishment of a basement membrane, cell–cell contacts between the migrating neural crest-derived cells and the vascular endothelial cells might contribute to the final fate and differentiation of both cells (13).

We demonstrate in an *in vitro* model that endothelial cells from the adrenal medulla can specifically modulate the differentiation of PC12 cells toward the chromaffin cell-like phenotype. The differentiating signals involve heterotypic cell–cell contacts and also humoral factors. We base this conclusion on morphological and functional changes in PC12 cells cocultured with BAME cells. Some of these changes might indicate a functional change or maturation of the PC12 cells—for example, the increase in [Met]enkephalin contents or the reduced rates of proliferation concomitant with clustering. Other signals, such as the rapid, transient increase in c-*fos*, suggest a functional modulation of the cells and/or the beginning of a switch in the state of the cellular differentiation of PC12 cells into chromaffin-like cells (7–9, 18). Further evidence for this switch toward the chromaffin cell lineage is found in a small, but significant increase (by ≈15%) in the total catecholamine contents and in the secretory response of PC12 cells that are cultured for 4 hr on confluent monolayers of BAME cells (P.I.L. and A. Munoz, unpublished observations). Finally, in coculture with BAME cells, PC12 cells no longer extended neurites in response to NGF, which commonly is equated with their differentiation toward sympathetic neurons (4). This lack of a visible NGF response is not due to a down-regulation of the NGF receptor (27) but might reflect some functional changes in PC12 cells (22).

PC12–endothelial cell contact is accompanied by a transient increase in c-*fos* expression as previously described for the response of PC12 cells to stimulation by peptide hor-

mones (21). Recently it has been demonstrated that such rapid, transient changes in the transcriptional regulation of oncogenes precede the long-term phenotypic cellular responses to microenvironmental stimuli (22). Thus, changes in c-*fos* expression are believed to signal the onset of modulations in cellular function—e.g., mechanism of signal transduction—and/or in the state of cellular differentiation (18, 19, 21). Such changes in c-*fos* levels were not observed in PC12 cells treated with glucocorticoids (6, 7), suggesting that there might be several distinct pathways that will lead to PC12 cell differentiation toward a chromaffin cell-like phenotype. In our case, c-*fos* activation might reflect an initial functional modulation (e.g., augmented catecholamine contents and secretory response) that might be the prelude to the change in differentiation, as manifested in the increased [Met]enkephalin contents and/or the lack of NGF-induced neurite extension.

The interactions between PC12 and BAME cells *in vitro* were found to be bidirectional, as indicated for instance by the mutual reduction in the rate of cellular proliferation. Bidirectional signals between PC12 cells and endothelial cells were previously suggested from implantation of PC12 cells into the rat brain periaqueductal gray matter *in vivo* (28). Preliminary ultrastructural studies indicate that similar heterotypic cell–cell interactions occur between chromaffin cells and endothelial cells during the early stages of neonatal development in the adrenal medulla, in particular, while the basal laminar membrane surrounding the vasculature is not yet fully developed (P.I.L. and B. R. Unsworth, unpublished data).

The importance of cell–cell contacts during development is well established. Thus, homotypic intercellular contacts seem to be required for normal pattern formation (29). Heterotypic cell–cell contacts—e.g., between endothelial cells and pericytes—are quite ubiquitous during development and maintained in mature organs (30). Interactions between endothelial cells and astrocytes *in vivo* are pivotal for the proper development and maintenance of a functioning blood–brain barrier (31). Cell–cell contacts between endothelial cells and other parenchymal cells have also been reported—e.g., during the development of mouse spinal cord (32) and in the organogenesis of the retina in several species (33). During capillary growth in the mesentery of young rats, fibroblasts that contact endothelial cells will differentiate into pericytes (34).

Several studies have shown that endothelial cells are pivotal for the survival and functional differentiation *in vitro* of a variety of other cells, such as astrocytes (35), smooth muscle cells (36), hepatocytes (37), parathyroid cells (38), or pancreatic beta cells (P. C. Mathias and P.I.L., unpublished observations). Our data are in line with this increasing body of evidence that endothelial cells comprise an essential part of the local microenvironment that will influence cellular differentiation during organogenesis.

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