Pharmacological Stimulation of Chromaffin Cell Proliferation in the Adult Adrenal Medulla*

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Summary. Many strains of rats develop adrenal medullary hyperplasia and/or neoplasia after chronic administration of a wide variety of pharmacologic agents. The same changes may occur spontaneously in the course of aging. Current evidence suggests that chromaffin cell proliferation in adult rats is regulated by a combination of hormonal and neurogenic signals. Drugs associated with adrenal medullary tumors might act directly or indirectly on the hypothalamic-endocrine axis or the autonomic nervous system to stimulate chromaffin cell proliferation through mechanisms which normally adjust cell number to meet physiological needs. The increased cell turnover rate might be a prelude to other events leading to neoplastic transformation.

Normal adult chromaffin cells are generally believed to have little ability to proliferate. However, the adrenal medulla in many strains of rats develops diffuse and nodular hyperplasia and, in some instances, neoplasia, either spontaneously in the course of aging, or after prolonged exposure to a variety of drugs and other agents (TISCHLER and DELELLIS, 1988). These proliferative changes are of fundamental interest because they might shed light on mechanisms which normally promote or prevent proliferation of chromaffin cells and of other neuroendocrine cells during development or in adult life. They are also of potential importance to human medicine for two reasons. First, since they resemble the proliferative changes seen in the adrenal glands of patients with Multiple Endocrine Neoplasia syndromes (DELELLIS et al., 1986), they might provide an experimental model for studying the pathogenesis of those disorders. Second, they suggest that it might be possible to stimulate chromaffin cells to proliferate *in vitro* prior to transplantation for the treatment of Parkinson's disease (LIEBERMAN et al., 1989).

We have recently shown that normal adult rat chromaffin cells also proliferate, and that their rate of proliferation can be markedly increased by short-term administration of agents which in long-term experi-

ments are associated with the development of adrenal medullary tumors (TISCHLER et al., 1988). It is thus now possible to study ways in which mechanisms regulating proliferation are altered in the course of the development and progression of neoplasia.

This paper reviews current knowledge of factors which stimulate chromaffin cell proliferation, and of their possible interrelationships and mechanisms of action.

ENDOGENOUS AND EXOGENOUS FACTORS IN ADRENAL MEDULLARY HYPERPLASIA AND NEOPLASIA

The development of adrenal medullary hyperplasia and neoplasia is affected both by endogenous and exogenous factors (TISCHLER and DELELLIS, 1988). The former, in order of importance, include the animals' strain, age and sex. Spontaneous proliferative changes are frequent in Wistar-derived and Fischer-344 rats, and infrequent in Osborne-Mendel and Charles River Sprague-Dawley rats. Within any given strain, proliferative changes occur most frequently in older animals and in males. Exogenous factors associated with adrenal medullary hyperplasia and neoplasia are summarized in Table 1.

To the extent that endogenous and exogenous factors are combined in an individual animal, a greater or lesser likelihood of hyperplasia and neoplasia might be expected. For example, both reserpine-induced and spontaneous hyperplasia or neoplasia are common in male Fischer 344 rats, but not in females (DIENER 1988). The incidence of hyperplasia and neoplasia can be further increased by dietary factors including excess food intake (GILBERT et al., 1958) and excessive intake or absorption of calcium (ROE and BÄR, 1985, BÄR, 1988).

Limited quantitative data which are available sug-

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Table 1. Agents associated with proliferative changes in the rat adrenal medulla

Agents	Reference
Agents Affecting Hypothalamic-endocrine axis	
Growth hormone	MOON et al. (1950), LUPULESCOU (1961)
Estrogen	LUPULESCOU (1961)
Antithyroid drugs	MARINE and BAUMANN (1945), STOLL et al. (1982)
Neuroleptics	ROE and BÄR (1985)
Alloxan	GRASSO (1963)
Agents affecting autonomic nervous system	
Nicotine	ERÄNKÖ et al. (1955)
Reserpine	DIENER (1988)
Timolol	DIENER (1988)
Dietary factors	
Excess food	GILBERT et al. (1958)
Excess Ca ⁺⁺ , Vit. D, Sugars, Starches, Sugar alcohols	ROE and BÄR (1985), BÄR (1988)
Miscellaneous	
Diphenylamine (antioxidant)	THOMAS et al. (1967)
Gemfibrozil (hypolipidemic)	FITZGERALD et al. (1981)
Zomepirac (anti-inflammatory)	MOSHER and KIRCHER (1988)
Retinol Acetate	KUROKAWA et al. (1985)
Ethylene glycol monoethyl ether	BROWN (1985)*
Radiation	WARREN et al. (1966)

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gest that the evolution of adrenal medullary proliferative changes from mild, diffuse hyperplasia through diffuse and nodular hyperplasia and neoplasia in rats is accompanied by increased production of norepinephrine, and increased norepinephrine-to-epinephrine ratios. Adrenal medullary neoplasms arising under diverse circumstances appear to produce almost exclusively norepinephrine (ERÄNKÖ, 1955; WARREN et al., 1966; MANGER et al., 1985; TISCHLER et al., 1985; BÄR, 1988) and, further, contain secretory granules which are smaller than those in normal chromaffin cells (TISCHLER et al., 1985; TISCHLER and DELELLIS, 1988). These findings initially suggested the possibility that proliferative capacity in the rat adrenal might be restricted to morphologically and functionally distinctive stem cells, possibly the small granule containing cells (TISCHLER et al., 1985). However, diffuse hyperplasias were known to contain typical E- and NE-type cells, suggesting either that the progeny of dividing stem cells initially mature into typical E- and NE-type cells, or that typical E- and NE-type cells proliferate to give rise to adrenal medullary hyperplasia, and that other factors are responsible for the distinctive characteristics of adrenal medullary neoplasms. To resolve this issue, we have studied chromaffin cell proliferation in normal adult rats and in rats treated for short periods with reserpine, one of the first agents implicated in the development of adrenal medullary tumors (DIENER, 1988).

CHROMAFFIN CELL PROLIFERATION IN THE NORMAL ADRENAL MEDULLA

Proliferation of chromaffin cells in the adrenal medulla of rats declines rapidly after birth. Mitotic figures are readily identified in routine, random histologic sections of neonatal rat adrenal, but are absent or extremely rare after the age of 60 days (JACKSON, 1919). For that reason, adult rat chromaffin cells were generally regarded for many years as essentially postmitotic. The issue was reassessed by MALVALDI et al. (1968) who serially sectioned adrenal glands of Wistar rats treated with colchicine, and concluded that small numbers of chromaffin cells do divide in adults. It was not, however, determined whether or not proliferation was restricted to a distinctive subset of cells. Using a similar strategy, we have shown mitoses in both mature-appearing E- and NE-type cells of normal control rats (TISCHLER et al., 1988) (Fig. 1). Animals receiving a single daily injection of reserpine (5–20 mg/kg) show a dose-dependent, up to eight-fold increase in mean mitotic count normalized per unit area of medulla occupied by chromaffin cells. This increase is observed after five days of treatment, but not after one day. It is observed in strains of rats with both high and low frequencies of adrenal medullary tumors, suggesting that it is a normal physiological response not predictive of proclivity toward tumor development (TISCHLER et al., 1989). Up to 35

Fig. 1. Electron micrograph of a mitotic chromaffin cell in a normal adult rat adrenal after fixation in glutaraldehyde and post-fixation in osmium tetroxide (COUPLAND and HOPWOOD, 1966). Numerous E-type granules are present in the dividing cell and its neighbors. $\times 4,700$

mitoses may be observed in a single histologic section in maximally stimulated animals six hours after final injection of reserpine plus colcemid.

Both mitoses and S-phase nuclei may be demonstrated simultaneously by addition of bromodeoxyuridine (BUdR) to this final injection, followed by immunocytochemical staining with a monoclonal anti-BUdR (GRATZNER, 1982; SCHUTTE et al., 1987). Under these circumstances, up to eight S-phase chromaffin cells may be observed in a single section of control adrenal, and up to 65 per section in a maximally stimulated gland (Figs. 3, 4).

Since typical appearing E- and NE-type cells do divide, it is likely that their sustained proliferation gives rise to diffuse adrenal medullary hyperplasia. The reasons for the distinctive characteristics of cells in adrenal medullary tumors remain unclear. We are

currently investigating the possibility of a genetic rearrangement in the vicinity of the phenylethanolamine-N-methyltransferase gene.

SIGNALS WHICH REGULATE CHROMAFFIN CELL PROLIFERATION

Current data suggest that chromaffin cell proliferation in the adult adrenal is mediated by the interaction of neurogenic and hormonal signals. In our ultrastructural study, reserpine-treated chromaffin cells showed varying degrees of secretory granule depletion, as also reported by others (ELVIN, 1968). Dividing chromaffin cells, however, contain few secretory granules, and contain numerous cytoplasmic vacuoles which might represent residua of recycled chromaffin granule membranes after exocytosis (Fig. 2). Both of

Fig. 2. Electron micrograph of a mitotic chromaffin cell in adrenal of an adult rat injected with reserpine (10 mg/kg/day) for 5 days. The cell is markedly depleted of granules and contains numerous vacuoles. The remaining granules are predominantly E-type, although some of the NE-type are also present. The adjacent cells show varying degrees of granule depletion. $\times 4,400$

these findings are consistent with intense secretory activity. Reserpine is known to directly deplete catecholamine stores. The resulting fall in blood pressure causes a reflex increase in the activity of the splanchnic nerve endings innervating the adrenal medulla, to stimulate both secretion and synthesis of catecholamines and other secretory granule constituents (SIETZEN et al., 1987). Since the dividing chromaffin cells show the greatest evidence of secretory activity, it is most likely that the neurogenic signals which stimulate chromaffin cell function after reserpine administration are also able to stimulate cell proliferation to meet increased physiological demands. Reserpine might also exert a number of direct effects including sensitization to Ca^{++} (JUREVICS and CARRIER, 1973), which may itself be a mitogenic signal (ALKON and RASMUSSEN, 1987). We have, however, not observed direct mitogenic effects of reserpine *in vitro*.

Neurogenic stimulation might also be a common denominator by which the apparently diverse agents in Table 1 cause adrenal medullary hyperplasia. Nicotine directly stimulates nicotinic cholinergic receptors in the adrenal, but more intense stimulation might actually result from indirect, reflexive stimulation in response to its various stressful systemic effects. Long-term treatment with anti-thyroid drugs might likewise act by this mechanism, since chronically hypothyroid rats are known to show increased activity of the sympathetic nervous system (WILSON and FOSTER, 1985). Other drugs and toxins, as well as radiation, probably also serve as systemic stressors. It is of interest that exposure to cold increases tritiated thymidine incorporation in the adult rat adrenal medulla (MALVALDI et al., 1972), suggesting that chromaffin cell proliferation in adults is normally regulated by neurogenic signals resulting from physical as well as pharmacological stressors.

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Fig. 3. Adrenal medulla from a normal adult rat injected with BUdr and colcemid 6 h before sacrifice. The section was stained immunocytochemically for phenylethanolamine N-methyltransferase to demonstrate epinephrine-producing cells (dark cytoplasm) (LLOYD et al., 1986), then stained again for BUdr using CoCl_2 to change the color of the reaction product (SCHUTTE et al., 1987). BUdr-labelled nuclei are evident in both E-type (*large arrow*) and NE-type (*small arrow*) cells. $\times 470$

Fig. 4. Adrenal medulla from an adult rat injected with reserpine (20 mg/kg/day) for 5 days, and with BUdr plus colcemid 6 h before sacrifice. Immunocytochemical staining for BUdr reveals numerous labelled nuclei scattered throughout the tissue. $\times 120$

In contrast, neurogenic signals probably play little role in stimulating chromaffin cell proliferation during early development or in adrenal tumors. The onset of functional splanchnic innervation in the rat is known to occur after the first week of life (SLOT-KIN, 1980), and adrenal medullary tumors are known to lose their nerve supply (TISCHLER et al., 1985). Under these circumstances, humoral factors might be of greater importance.

Compelling evidence for a role of pituitary hormones in regulating chromaffin cell proliferation is provided by a report suggesting that hypophysectomy might prevent the development of adrenal medullary tumors in a susceptible rat strain (MANGER et al., 1982). The normal adrenal medulla contains receptors for insulin-like growth factors (IGF's) (DAHMER and PERLMAN, 1988a). Since levels of circulating IGF-1 (somatomedin C) are regulated by growth hormone, IGF-1 might be a common denominator explaining both the requirement for an intact pituitary in development of spontaneous tumors, and the increased frequency of such tumors after administration of exogenous growth hormone. IGF-1 has been shown to stimulate proliferation of PC12 pheochromocytoma cells *in vitro* (DAHMER and PERLMAN, 1988b). Another pituitary hormone which might be indirectly implicated is prolactin. Growth hormone and prolactin are approximately 40% homologous, and in high concentrations growth hormone can affect prolactin receptors (FRIESEN, 1980). Exogenous estrogen, or the hyperestrogenic state resulting from obesity in overfed aged rats, might be expected to increase prolactin production, as might increased concentrations of thyrotropin releasing hormone which would result from treatment with antithyroid drugs (VISCARELLO, 1978). Certain neuroleptics might also increase prolactin production by decreasing dopamine release. Finally, prolactin release in rats is stimulated by acute stress (MINAMITANI et al., 1987), and increased serum prolactin levels in young rats with pituitary grafts are associated with increased synthesis and secretion of catecholamines (FERNANDEZ-RUIZ et al., 1987). Aged rats of some strains develop prolactin-producing pituitary tumors even more frequently than adrenal medullary tumors (BUREK, 1978; THOMPSON et al., 1981). Although all animals with proliferative changes in the adrenal medulla do not have morphologically apparent pituitary lesions, at least some with anatomically normal pituitaries might have hypothalamic defects resulting in decreased dopaminergic inhibition of prolactin release (SARKAR et al., 1982). Any effects of prolactin on chromaffin cell proliferation are likely to be indirect,

because the normal rat adrenal medulla has no demonstrable prolactin receptors (COSTLOW and MCGUIRE, 1977). We have also been unable to demonstrate prolactin binding to PC12 pheochromocytoma cells (KAPLAN and TISCHLER, unpublished data).

Growth factors which are not related to classic pituitary hormones may also affect chromaffin cell proliferation. NGF (LILLIEN and CLAUDE, 1985) acidic FGF (CLAUDE et al., 1987) and basic FGF (STEMPLE et al., 1988) are transiently mitogenic in cell cultures of neonatal rat adrenal. Epidermal growth factor (EGF) is not mitogenic under the same conditions (LILLIEN and CLAUDE, 1985). It is of interest that the pituitary is a rich source of FGF (STEMPLE et al., 1988).

TRANSDUCTION MECHANISMS FOR MITOGENIC SIGNALS

The mechanisms by which neurogenic and hormonal signals induce cell proliferation in the adrenal medulla are unknown, but probably involve interactions at multiple levels between several intracellular signal transduction pathways. One of these might involve the *c-fos* oncogene. It has recently been shown that depolarization of PC12 pheochromocytoma cells after cholinergic stimulation or exposure to depolarizing concentrations of K^+ causes a large, rapid, transient induction of *c-fos* (GREENBERG et al., 1988). This activation is calcium-dependent, and is inhibited by the calcium chelator EGTA. Similar activation, which is not calcium-dependent, occurs after exposure to NGF or EGF (GREENBERG et al., 1985). These growth factors are not normally mitogenic for PC12 cells, although NGF is mitogenic for certain PC12 cell mutants (BURSTEIN and GREENE, 1982) and for PC12 transfectants which overexpress the *myc* oncogene (MARUYAMA et al., 1987). The protein product of the *fos* oncogene is able to interact with that of the *jun* oncogene (RAUSCHER et al., 1988), which probably regulates cell proliferation and other functions by binding to regulatory regions of DNA. It is intriguing to speculate that the activation of *c-fos* by neurogenic stimuli or growth factors might lead to cell proliferation only when accompanied by appropriately timed permissive events, such as activation of other oncogenes, which are hormonally mediated (WHITMAN et al., 1986). An increase in intracellular Ca^{++} concentration might also be required (ALKON and RASMUSSEN, 1988). Under other circumstances, *c-fos* probably plays a role in regulating neuronal and neuroendocrine function (MORGAN et al., 1987).

We have recently shown that depolarization of a variety of other neuroendocrine tumor cell lines also

activates c-fos (HOFFLER et al., 1987). Since most neuroendocrine cells possess voltage-gated ion channels (TISCHLER et al., 1976a, b), Ca^{++} flux and oncogene activation subsequent to opening of these channels by depolarizing stimuli might together constitute one general mechanism whereby physiological signals which stimulate neuroendocrine function might also stimulate proliferation of neuroendocrine cells in normal or pathological states.

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