SHORT COMMUNICATION

Comparative Studies of Chromaffin Cell Proliferation in the Adrenal Medulla of Rats and Mice

Spontaneous and drug-induced pheochromocytomas are common in rats and rare in mice. The antihypertensive drug reserpine has been shown to both induce pheochromocytomas and stimulate chromaffin cell proliferation in rats, leading to the hypothesis that reserpine causes pheochromocytomas indirectly by providing a proliferative setting in which DNA damage may occur. The present investigation was undertaken to obtain baseline information on the relationship across species between chromaffin cell proliferation and pheochromocytomas. Basal chromaffin cell proliferation was compared in age-matched young adult mice and rats. In addition, mice were studied for adrenal medullary responses to reserpine, and mouse chromaffin cells in vitro were studied for responses to agents that are mitogenic for cultured rat chromaffin cells. Concurrently maintained F-344 rats and several strains of mice showed no significant difference in basal BrdU incorporation over a 1-week period. Mice also showed an adrenal medullary proliferative response to reserpine that was comparable to the response previously reported for rats. However, there was a marked disparity between rat and mouse chromaffin cells in vitro, and cultured mouse chromaffin cells did not respond to any mitogens. The in vivo data indicate that interspecies differences in basal- or reserpine-stimulated chromaffin cell proliferation sufficient to account for different frequencies of pheochromocytomas are not detectable at a single time point in young adult animals. However, the possibility that such differences might emerge with aging has not been ruled out. These data further suggest that stimulation of chromaffin cell proliferation might be necessary but not sufficient for development of pheochromocytomas or that stimulated proliferation in mice might not be sustained. The inability of cultured mouse chromaffin cells to respond to mitogens raises the speculation of whether mechanisms that prevent proliferation of normal chromaffin cells in vitro might also help to protect mice from developing pheochromocytomas.

Spontaneous proliferative lesions of adrenal chromaffin cells are common in aging laboratory rats. These lesions, which include diffuse hyperplasia, nodular hyperplasia, and pheochromocytomas, also occur frequently in rat carcinogenicity studies, and their induction appears to reflect exacerbation of proclivities toward their spontaneous development in susceptible rat strains (Tischler and DeLellis, 1988; Strandberg, 1996). The lesions occur most frequently in male rats, with reported lifetime frequencies of over 80% in the Wistar strain and over 30% in the Fisher 344 and Sprague–Dawley strains.

In contrast to rats, mice seldom develop adrenal proliferative lesions. Pheochromocytomas occur spontaneously with reported lifetime frequencies of 3% or less in most studies involving mice, and their frequency is usually not affected by xenobiotic agents (Frith, 1983; Longear, 1996; Tischler and Sheldon, 1996). Substances that induce pheochromocytomas in rats are pharmacologically diverse and, for the most part, nongenotoxic. These have recently been reviewed (Strandberg, 1996). It has been hypothesized that a common denominator in their mode of action is stimulation of chromaffin cell proliferation by neurally derived signals that also regulate catecholamine production and release. This mitogenic response, which might provide a means for long-term adjustment of adrenal medullary responses to increased physiological demand, could also facilitate the occurrence of mutations or other genetic damage. Evidence to support this hypothesis is provided by studies showing that agents associated with development of pheochromocytomas in rats are mitogenic for chromaffin cells in vivo. One of the more important of these agents is the antihypertensive drug reserpine (Tischler et al., 1991).

Reserpine was found to be associated with both adrenal medullary hyperplasia and pheochromocytomas in rats, but not mice, in an NCI bioassay (Department of Health, Education and Welfare, 1979). Subsequent studies showed that short-term administration of reserpine to rats causes an increase in chromaffin cell proliferation that is eliminated by adrenal denervation (Tischler et al., 1991). In addition, agents that mimic neural stimulation by activating intracellular signaling pathways known to mediate the effects of acetylcholine or of peptide neurotransmitters were shown to stimulate proliferation of rat chromaffin cells in cell culture (Tischler et al., 1994). These in vitro mitogens include phorbol esters, which mimic muscarinic cholineric stimulation by activating protein kinase C, and forskolin, which mimics peptidergic stimulation by activating adenylate cyclase. The peptide growth factors, nerve growth factor (NGF) (Lillien and Claude, 1985) and fibroblast growth factor (FGF) (Manthanthappa et al., 1990), which may activate portions of the...
same signalling pathways (Tischler et al., 1994), are also mitogenic for rat chromaffin cells in vitro.

Although mouse chromaffin cells have been known for some time to proliferate in adult life (Messier and Leblond, 1960; Benedetti, 1975; Jurecka et al., 1978), it is not known whether differences in the frequency of pheochromocytomas between rats and mice can be explained simply on the basis of differences in chromaffin cell proliferation. In this study, we have evaluated basal and reserpine-stimulated proliferation of chromaffin cells in young adult mice and rats using 5-bromo-2′-deoxyuridine (BrdU) incorporation as a proliferation marker. We have also studied the responses of cultured mouse chromaffin cells to agents known to be mitogenic for male and female rat chromaffin cells under the same culture conditions.

MATERIALS AND METHODS

Animals, BrdU labeling, and drug administration. A 1-week study was conducted to assess strain and sex differences in basal rates of adrenal chromaffin cell proliferation in mice and to compare chromaffin cell proliferation in mice and rats. This experiment was performed with male Charles River B6C3F1, C3H/He, CD-1, and male and female C57BL/6 mice and concurrently maintained male Charles River F-344 rats, using methods that we have previously described in detail (Tischler et al., 1991, 1995). Briefly, six mice of each strain or sex and six male rats, all 10 to 12 weeks of age, were obtained from Charles River Research Laboratories (Raleigh, NC) and acclimated for approximately 2 weeks prior to the start of the experiment. Alzet osmotic pumps (Alza Corp., Palo Alto, CA; Model No. 2ML2) containing 20 mg/ml BrdU (Sigma Chemical Co., St. Louis, MO; Cat. No. B5002) were then implanted intraperitoneally. BrdU, which is incorporated into S-phase nuclei in place of thymidine, serves as a marker for DNA replication (Gratzner, 1982). The animals were killed after 1 week of BrdU labeling. The adrenal glands were immediately removed and fixed for 24 hr in 10% buffered formalin, pH 7.0, dehydrated in a graded ethanol series, and embedded in paraffin. Five-micrometer-thick sections were prepared for immunohistochemistry. Additional BrdU-labeled adrenal glands were obtained from a group of male F-344 rats that were control animals in a cell proliferation experiment conducted identically to the study described above, but not run concurrently.

A separate 1-week study of the effects of dietary reserpine on proliferation of mouse chromaffin cells was conducted using adult male C57BL/6 mice. Twenty-four mice that were 10 to 12 weeks of age (Taconic Farms, Germantown, NY) were divided into two groups receiving either a control diet or 10 ppm reserpine as a dietary admixture, similar to that described in the NCI Bioassay of Reserpine for Possible Carcinogenicity (Department of Health, Education and Welfare, 1979). The admixture was prepared by mixing reserpine with standard laboratory chow (Purina Certified Rodent Chow 5002, meal), as previously described (Tischler et al., 1991, 1995). The administered dose of reserpine was previously shown to be associated with pheochromocytomas in rats, but not in male or female mice, in the NCI bioassay, and to stimulate chromaffin cell proliferation in rats (Tischler et al., 1991, 1995). Osmotic pumps containing 20 mg/ml BrdU were implanted at the start of the experiment, and the animals were maintained for 1 week as described above, except for being housed two per cage.

Tissue analysis. Deparaffinized histologic sections were subjected to two 5-min pulses of heating in a 1400-W Kenmore microwave oven (Sears Roebuck and Co., Chicago, IL) in 0.01 M citrate buffer, pH 6.0 (Shi et al., 1991), followed by immunohistochemical staining for BrdU as previously described (Tischler, 1995). After staining for BrdU, sections were stained for tyrosine hydroxylase (TH), and, in some instances, also for phylethano-

lamine N-methyltransferase (PNMT), as previously described (Tischler, 1995). Immunoreactivity for TH, the rate-limiting enzyme in catecholamine synthesis, identifies all chromaffin cells, while PNMT, the enzyme that synthesizes epinephrine (E) from norepinephrine (NE), serves as a marker for E cells. NE cells can be identified by the absence of immunoreactive PNMT (Verhoeff et al., 1985). For each animal, all chromaffin cells in a single section of adrenal showing the greatest area of medulla were counted visually and scored for the presence or absence of BrdU labeling. BrdU labeling indices were calculated as percentages of total chromaffin cell number for each population. Differences between groups were statistically evaluated as described in the table legends.

Cell culture studies. Pooled adrenal medullary tissue from 6- to 8-week-old male C57BL/6 mice and age-matched male F344 rats (Taconic Farms) was dissociated in collagenase followed by trypsin, according to the protocol that we have previously employed for dissociation of rat adrenal medulla, and chromaffin cell proliferation was studied, as previously described (Tischler et al., 1992). Briefly, the dissociated cells were plated in 35-mm collagen-coated tissue culture dishes in RPMI 1640 medium with 10% heat-inactivated horse serum and 5% fetal bovine serum, with 10 μM BrdU. Approximately 1000 viable chromaffin cells were plated per dish. The cultures were maintained for 5 days at 37°C, either in medium supplemented with substances previously shown to be mitogenic for rat chromaffin cells under the same conditions or in control medium, and then fixed and stained for both TH and BrdU, as previously described (Tischler et al., 1992). Known mitogens for adult rat chromaffin cells were NGF (50 ng/ml) (2.5 S mouse salivary gland NGF, prepared as described by Bocchini et al. (1969) (Tischler et al., 1993, 1995), basic fibroblast growth factor (bFGF, 40 ng/ml) (recombinant human bFGF; Calbiochem, San Diego, CA) (Manthanthappa et al., 1990), phorbol myristate acetate (PMA, 50 μM; Sigma Chemical Co.) (Tischler et al., 1995), and forskolin (5 μM; Sigma Chemical Co.) (Tischler et al., 1995). In addition, some cultures were supplemented with substances that we have previously found not to be mitogenic for rat chromaffin cells, but regarded as potential mouse chromaffin cell mitogens because of their other actions on a variety of cell types. These included epidermal growth factor (EGF, 50 ng/ml) (Kilpatrick and Bartlett, 1995), while CNTF, which was not mitogenic for mouse chromaffin cells, but regarded as potential mouse chromaffin cell mitogens because of their other actions on a variety of cell types. These included epidermal growth factor (EGF, 50 ng/ml), neurotrophin 3 (NT3, 10 ng/ml), neurotrophin 4 (NT4, 50 ng/ml), and ciliary neurotrophic factor (CNTF, 10 ng/ml). BDNF, NT3, NT4, and CNTF were recombinant human proteins provided by Dr. Chitra Suri of Regeneron Pharmaceuticals (Tarrytown, NY). Because CNTF may act synergistically with other growth factors (Ip et al., 1994), each of the conditions was tested both alone and in combination with CNTF.

PRELIMINARY dose–response curves for NGF (5–100 ng/ml), forskolin (1–50 μM), and PMA (10 nM–1 μM) revealed no dose-dependent stimulation of mouse chromaffin cells by those agents, and the agents were therefore definitively studied at concentrations previously found optimal for rat chromaffin cells. The neurotrophic peptides have similar activities across species and were used at concentrations that were likely to be saturating based on other studies. Basic FGF, for example, exerts maximal mitogenic effects on neuroepithelial cells from the mouse CNS at concentrations from 5 to 50 ng/ml (Kilpatrick and Bartlett, 1995), while CNTF, which was not mitogenic for either rat or mouse chromaffin cells, exerts maximal survival-promoting effects on rat CNS glia at concentrations from 5 to >100 ng/ml (Louis et al., 1993). BrdU labeling indices were calculated from counts of approximately 300 consecutively scored TH-positive cells for each culture condition.

RESULTS

In Vivo Studies

Basal proliferation. There was no significant difference in basal BrdU labeling of chromaffin cells between concurrently maintained rats and mice. Mean percentages of la-
belled cells ranged from 0.96 ± 0.18 to 1.40 ± 0.27 across strains and species (Table 1). Individual rat adrenals contained from 29 to 59 labeled nuclei per cross section vs 7 to 47 in individual mouse adrenals, due to the greater cross-sectional area of medulla in rats. In the separate group of rats from a previous experiment, 3.20 ± 0.30% of chromaffin cells were labeled with BrdU.

Response to reserpine. Reserpine administration to mice caused an increase in chromaffin cell proliferation comparable to that previously reported in rats (Tischler et al., 1991, 1995). The control C57BL/6 mice in this experiment showed 4.4 ± 0.7% of chromaffin cells labeled with BrdU during the 1-week labeling period vs 10.8 ± 1.3% labeled in animals ingesting 10 ppm reserpine (Table 2). Both E and NE cells were labeled in both groups.

Cell Culture Studies

There were no significant effects detected with any agents tested on mouse chromaffin cells in vitro, while concurrently maintained rat chromaffin cells exhibited typically robust responses to the previously reported mitogens. Agents not known to be mitogenic for rat chromaffin cells also failed to significantly affect cells from mice (Table 3). Between 0 and 3.1% of mouse chromaffin cells stained for BrdU after the 5-day labeling period vs up to 50% for their rat counterparts. From 0 to 14 individual labeled chromaffin cells were scored in the mouse cultures vs 3 to 168 labeled cells in cultures from rats.

DISCUSSION

In the 2-year National Cancer Institute bioassay of reserpine for possible carcinogenicity, adrenal medullary lesions classified as nodular hyperplasia and/or pheochromocytoma developed in 17% of male F344 rats and in 0% of male B6C3F1 mice. The corresponding figures for animals ingesting 10 ppm reserpine were 49% for rats and 1% for mice (Department of Health, Education and Welfare, 1979). The present study was undertaken with two objectives: to determine whether the dramatic interspecies difference in tumor frequency can be straightforwardly explained by equally dramatic differences in baseline or reserpine-stimulated chromaffin cell proliferation and to determine whether the types of signals that drive proliferation of chromaffin cells from the two species are similar in vitro.

The basal rate of BrdU incorporation in concurrently maintained, age-matched male F344 rats and several strains of mice was found to be virtually identical. Although the percentage of rat chromaffin cells labeled with BrdU in this side-by-side comparison was somewhat lower than that in a previous experiment of ours or in studies reported by others (Verhofstad, 1993), even the cross-experiment differences between species were small. The present findings therefore suggest that the markedly different frequencies of pheochromocytomas in control rats and mice cannot be simply explained by differences in chromaffin cell proliferation that become evident early in life.

More important than the basal comparison of mice and rats was the finding that chromaffin cells in mice show a proliferative response to reserpine comparable to that previously reported in rats. Mouse chromaffin cells have not previously been shown to proliferate in response to reserpine administration. Although reserpine is mitogenic in both species, it causes pheochromocytomas only in rats (Department of Health, Education and Welfare, 1979). This disparity suggests that while stimulation of chromaffin cell proliferation may be necessary for induction of pheochromocytomas in rodents, it is not sufficient. An alternate explanation is that...
mouse chromaffin cells stimulated with reserpine do not exhibit the sustained proliferative response associated with tumor induction by other nongenotoxic agents (Marsman et al., 1988). We have previously demonstrated that the reserpine response in rats persists for at least 12 weeks (Tischler et al., 1995).

It must be borne in mind that these in vivo studies were conducted in young animals and that only a single age group was examined. Rats have been shown to exhibit age-dependent changes in neural stimulation of the adrenal medulla and in adrenal medullary responses to stress (Ito et al., 1986). The possibility that large interspecies differences in basal or reserpine-stimulated chromaffin cell proliferation emerge later in life has not been ruled out and will require long-term study.

Numerous previously reported experiments have demonstrated robust proliferation of adult rat chromaffin cells in vitro (Mananthappa, 1990; Tischler et al., 1994). The failure of cultured mouse chromaffin cells to respond to agents that are mitogenic for their rat counterparts is unexplained. However, the same result was obtained from multiple experiments. Responses to other candidate mitogens were also not demonstrable. While the possibility of small responses to some agents has not been entirely ruled out, the responses would have to be at least an order of magnitude lower in cells from mice than of rats. Although this interspecies difference might be viewed as a cell culture artifact, the possibility that mechanisms which brake proliferation of mouse chromaffin cells in vitro might also protect mice from pheochromocytomas may be speculatively raised. This speculation is plausible because denervation, which is known to profoundly influence chromaffin cell function (Sietzen et al., 1987), occurs both when cells are cultured and during the development of pheochromocytomas (Tischler and DeLellis, 1988). Possible effects of denervation include altered expression of receptors for autocrine or paracrine growth factors known to be present in chromaffin cells (Meissinger et al., 1996), altered expression of the growth factors themselves, or activation of mitotic checkpoints.

An important incidental finding in these studies was that a two- to threefold difference in basal labeling of chromaffin cells with BrdU may be observed between experiments, even when the animals are maintained under identical conditions, as in the two groups of rats that we examined. Even larger differences might be attributable to differences in animals maintained, such as group housing (Table 2) versus solitary housing (Table 1), as in our two experiments with male C57BL/6 mice. This finding suggests that interexperimental comparisons should be made with caution and emphasizes that the recently estimated renewal rate of about 1% per day for adult rat chromaffin cells (Verhofstad, 1993) is an approximation.

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REFERENCES


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