Angiotensin-Converting Enzyme Inhibition Reduces Neuroblastoma Cell Growth Rate (43189)

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Abstract. **Because of the known capacity of angiotensin II to serve as a growth factor in multiple tissues, we elected to study the effects of renin-angiotensin system inhibition on the growth of human SH-SY5Y neuroblastoma cells. Cells were treated with captopril (0.05–5 mg/ml), enalapril, or enalaprilat (0.02–5 mg/ml) or saralasin (0.1–0.25 mg/ml). In all cases, statistically significant reductions in cell growth were seen over 5 days of culture. In additional experiments, captopril and enalaprilat significantly decreased thymidine incorporation into DNA in these cells. The administration of angiotensin II in the presence of captopril partially offset these suppressive effects.**

[P.S.E.B.M. 1991, Vol 196]

The renin-angiotensin systems is a known determinant of vascular fluid homeostasis and blood pressure regulation. Over the last 10 or so years, abundant evidence has accumulated to indicate that local renin systems exist in many body tissues (1–5). In addition, some neuroblastoma cells have been reported to contain all components of the renin-angiotensin system (6, 7).

More recently, it has been shown that angiotensin II is capable of acting as a growth factor for appropriate target tissues (8–12). For example, angiotensin II appears to produce hypertrophy of arterial smooth muscle cells, although it may produce hyperplasia of subsets of vascular cells (9, 12). In addition, angiotensin II, depending on the state of the target cells, can cause either mitogenesis or differentiation in cultured adrenal glomerulosa cells (10, 11). The link between angiotensin II and cell growth was further strengthened by the finding that the *mas* oncogene may code an angiotensin II receptor (13).

In view of these reports, we hypothesize that the intrinsic renin-angiotensin system that has been identified in some neuroblastoma cell lines may serve as an autocrine or paracrine growth-regulating system for these cells. To test this hypothesis and the more global hypothesis that angiotensin may regulate the growth of other classes of malignant cells, we explored the effects of converting enzyme inhibition on cultured human neuroblastoma cells.

Materials and Methods

SH-SY5Y human neuroblastoma cells, a subclone of SK-N-SH cells, were obtained from Dr. Om Prakash of this institute. Cells (10^4/ml × 2 ml) were cultured in 35-mm petri dishes in a medium containing minimum essential medium supplemented with 10% fetal bovine serum (Gibco), 160 units of penicillin G/ml, and 0.16 mg of streptomycin/ml. Either additional medium or medium containing captopril (0.05–5 mg/ml final concentration; E. R. Squibb & Sons), enalapril, or enalaprilat (0.02–5 mg/ml final concentration; Merck, Sharpe & Dohme) were added to the cells. Daily cell counts were performed (six to eight dishes per point) in each group and daily all plates received fresh medium with or without test drugs. Cells were examined daily to assess viability by light microscopy and trypan blue exclusion. Growth curves were constructed over a 5-day period. Also, the effects on cell growth of saralasin (0.10–0.25 mg/ml; Sterling Drug) and bovine insulin (1.0 mg/ml; Sigma) were studied using the same protocol.

In additional experiments, 2×10^4 cells/200 μl were plated in each microwell of a flat-bottomed 96-well cell culture cluster dish (culture medium as described above) in the presence or absence of captopril

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Received May 29, 1990. [P.S.E.B.M. 1991, Vol 196]
Accepted October 17, 1990.

0037-9727/91/1963-0280$3.00/0

or enalaprilat at the concentrations mentioned above; 1.0 μCi of [*methyl*-^{3}H]thymidine (New England Nuclear) was then added to each well and incubation was carried out over 24 hr. At the end of 24 hr, thymidine incorporation into DNA was determined using standard methodology and a LKB 1295-001 cell harvester (14).

To determine whether the growth-inhibiting effects of converting enzyme inhibition were related to lowered angiotensin II concentrations in the cultures, thymidine incorporation studies were performed as described above in the presence or absence of (i) various converting enzyme inhibitors and (ii) 10^{-5} or 10^{-6} M angiotensin II. [^{3}H]Thymidine incorporation was assessed at the end of 12 hr.

Statistics

Data were analyzed by the Student's *t* test. Where indicated in the text, a one-tailed test was used. Where not specifically indicated, a two-tailed test was employed.

Results

Each of the converting enzyme inhibitors tested resulted in decreased neuroblastoma cell growth rate. In the case of captopril, this suppression of cell growth reached statistical significance at the end of the study (Day 5) at the 1.0 and 5.0 mg/ml concentrations (Fig. 1). Similarly, in the case of enalaprilat, statistically significant suppression of growth was seen at the 5.0 mg/ml and the 1.0 mg/ml concentrations when a one-tailed *t* test was used (Fig. 2). Finally, enalapril at both the 1.0 and 5.0 mg/ml concentrations resulted in statistically significant suppression of cell growth; indeed, the 0.1 mg/ml concentration produced statistically significant reduction in cell number at most time points (two-tailed test) and even at Day 5 when analyzed by a one-tailed *t* test (Fig. 3).

Although insulin at 1 mg/ml had essentially no effect on cell number, saralasin, a competitive angiotensin II antagonist, at concentrations ranging from 100 to 250 μg/ml did blunt cell growth ($P < 0.01$, Fig. 4).

Cell growth resumed when converting enzyme inhibitor-containing medium was removed from cells grown in its presence for 5 days and replaced with fresh medium not containing test drugs. No morphologic evidence of cell death was seen at any concentration of the drugs. Neither captopril nor enalapril at a concentration of 1 mg/ml produced a decrease in the growth of the simian T lymphocyte line MLA 144; at 5 mg/ml, each compound produced a small decrease in cell

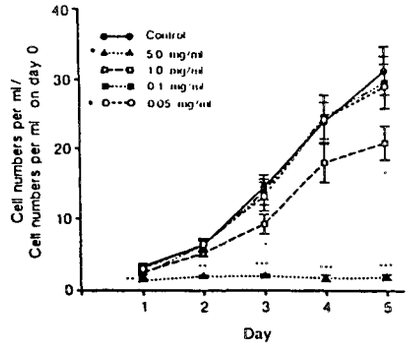

Figure 1. Effect of captopril on cell number. ***Implies $P < 0.001$, **implies $P < 0.01$, and *implies $P < 0.05$ when compared with control.

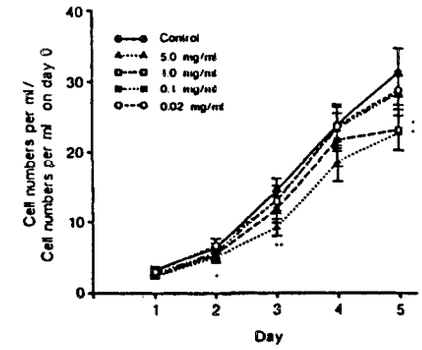

Figure 2. Effect of enalaprilat on cell number. *Implies $P < 0.05$ by one-tail *t* test, **implies $P < 0.05$ by two-tailed *t* test when compared with control.

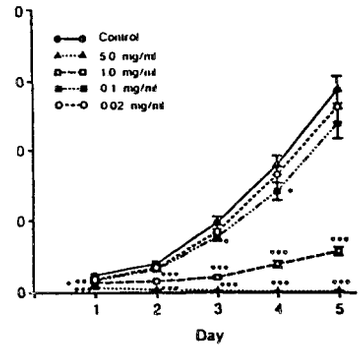

Figure 3. Effect of enalapril on cell number. ***Implies $P < 0.001$, **implies $P < 0.01$, and *implies $P < 0.05$.

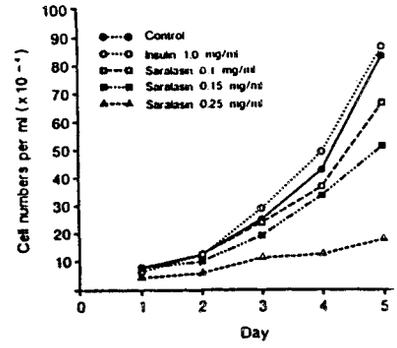

Figure 4. Effects of insulin and varying concentrations of saralasin on cell number.

growth, but to a much lesser degree than seen with neuroblastoma cells (data not shown).

Thymidine incorporation into DNA was reduced by captopril (Fig. 5). A similar effect was seen with enalaprilat, but only at the highest drug concentrations. Note that significant suppression of thymidine incorporation occurs when cells are exposed to 0.1 mg/ml of captopril.

As shown in Table I, the addition of angiotensin II to converting enzyme-treated cells partially offset the growth-inhibiting effects of converting enzyme inhibition. When 10^{-6} M angiotensin II was added to the incubation medium in the absence of converting enzyme inhibitors, a 19% increase (as compared with serum-containing medium alone) in thymidine incorporation into DNA was observed ($P < 0.01$).

Discussion

The demonstration of local renin systems in various tissues coupled with the increasing evidence to support the concept that angiotensin II is a growth factor led us to investigate the possibility that angiotensin II, acting in an autocrine, paracrine, or endocrine fashion, may play a role in the growth of malignant neoplasms (8–12). We initially chose to study a human neoplastic cell line analogous to the mouse line that has been associated with the *in situ* production of components of the renin-angiotensin system (6, 7). This was done because, if an autocrine mechanism is operative, one would anticipate that cells capable of synthesizing angiotensin II would most readily demonstrate the effects of the inhibition of its production. The first test cell line we examined was the SH-SY5Y human neuroblastoma cell line. In these cells, we found that both converting enzyme inhibitors and an angiotensin II antagonist inhibited cell growth, albeit at a high concentration.

Both captopril, a sulfhydryl-containing converting enzyme inhibitor, and enalapril, a nonsulfhydryl-containing converting enzyme inhibitor, produce clear reductions in cell growth. Thus, it does not appear that the effect we are observing are intrinsic to the chemical structure of either compound. Rather, the results we have obtained likely are related to the ability of these agents to inhibit converting enzyme. Moreover, it does not appear that either agent actually kills cells. We detected no evidence of cell death in our cultures and all cells resumed normal growth when test drugs were removed. Finally, the effects of converting enzyme inhibitors on cell growth appear to be cell-type specific in that little or no effect of these agents could be detected on the growth of MLA 144 cells.

As expected, thymidine incorporation into DNA fell with converting enzyme inhibition. Captopril at 0.1 mg/ml produced a significant decrease in thymidine incorporation into DNA. Saralasin, like converting enzyme inhibitors, blocked cell growth. Although the concentrations of peptide required were high, similar concentrations of insulin had no effect, making it unlikely that the observed action of saralasin was nonspecific. The fact that both an angiotensin II antagonist and a series of converting enzyme inhibitors decrease cell growth adds support to the concept that these agents are acting by interfering with angiotensin production, or reducing angiotensin II action, at an important site. This hypothesis is further supported by the finding that the administration of angiotensin II is capable of, at least partially, offsetting the growth-inhibitory effects of converting enzyme inhibition. Given the rapid degradation of angiotensin II in serum-containing culture medium, it is not surprising that full restitution of thymidine incorporation was not achieved. Finally, the observation that 10^{-6} M angiotensin II produced a significant increase in thymidine incorporation into DNA in these cells supports our hypothesis that angiotensin II is, in fact, a regulator of their growth.

If the hypothesis that the interruption of angiotensin II function accounts for these observations is correct, how can the high doses required and the unusual rank order of potency of these converting enzyme inhibitors be explained? We do not have a ready explanation, but wonder if the site of action of the drugs may be only poorly accessible to the tissue culture medium (for example, intracellular). Similarly, we know little of the half-lives, metabolism, or intracellular/extracellular partitioning of active converting en-

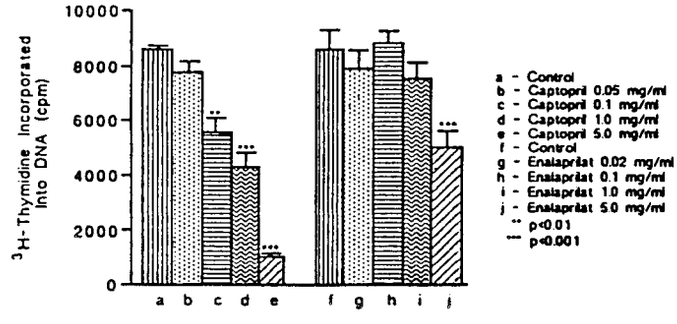

Figure 5. Effect of captopril and enalaprilat on [^{3}H]thymidine incorporation into DNA in SH-SY5Y cells.

Table I. [^{3}H]Thymidine Incorporation into DNA

cpm, Mean ± SEM				
Control	Captopril (1 mg/ml)	Captopril (1 mg/ml) and angiotensin II (10^{-5} *M*)	Enalapril (0.2 mg/ml)	Enalapril (0.2 mg/ml) and angiotensin II (10^{-6} *M*)
1851.9 ± 56.0	1381.5 ± 67.1[a]	1578.7 ± 60.9[b]	1080.2 ± 93.2[a]	1377.8 ± 92.3[c]

[a] Significantly different from control, $P < 0.001$.
[b] Significantly different from captopril-treated cells, $P < 0.01$.
[c] Significantly different from enalapril-treated cells, $P < 0.01$.

zyme inhibitors or pro-drugs (such as enalapril) in this system; thus, dose and potency rank order may not parallel those found in other systems.

Other possibilities should also be considered. For example, it may be that neuroblastoma cell growth is indeed modulated by angiotensin II, but that the enzyme responsible for the production of this peptide is not angiotensin-converting-enzyme but another moiety only inhibited by high concentrations of angiotensin-converting enzyme inhibitors. Further studies will be required to exclude this and other possible alternative explanations, as well as to demonstrate if angiotensin II is produced in an autocrine fashion by these neuroblastoma cells or is simply generated in the serum component of the culture medium. Nonetheless, the hypothesis of drug action at a poorly accessible site appears to be the most conservative explanation of these findings.

We have provided evidence to suggest that converting enzyme inhibition per se results in the decreased growth of at least one neuroblastoma cell line. We believe, because chemically distinct converting enzyme inhibitors and angiotensin antagonists suppressed growth, that the phenomenon we observed resulted from converting enzyme inhibition and a subsequent reduction in angiotensin II levels. Additional studies will be required to fully exclude other possibilities. However, on the basis of these studies, we suggest that converting enzyme inhibition may provide a novel therapy for patients suffering from otherwise untreatable neuroblastoma. The concentrations required to produce an effect in our system are not different from those that could be achieved with aggressive therapy in humans.

Additionally, these results suggest that converting enzyme inhibition may play a therapeutic role in the treatment of other malignancies and, in particular, those associated with intrinsic angiotensin-generating systems and/or angiotensin receptors. We, therefore, suggest that converting enzyme inhibition may play a role in the treatment of diseases as diverse as hepatoma, pituitary tumors, ovarian neoplasms, gliomas, Kaposi's sarcoma, and adrenal carcinoma.

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