Isolation and Characterization of Renin-expressing Cell Lines from Transgenic Mice Containing a Renin-Promoter Viral Oncogene Fusion Construct*

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We constructed transgenic mice containing a renin-promoter SV40 T antigen fusion transgene with the intention of inducing neoplasia in renin-expressing cells and isolating renin-expressing cell lines in vitro. We examined six kidney tumors from mice representing three different transgenic lines and found they expressed their endogenous renin gene. Initially, five noncncial kidney tumor-derived cell lines were established which expressed their endogenous renin gene in addition to the transgene. They retained active renin intracellularly and constitutively secreted an inactive form of renin (prorenin). One of these cell lines was cloned to homogeneity. This line maintained high level expression of renin mRNA throughout 3 months of continuous culture. Although the cells contained an equal proportion of active and inactive renin, the species constitutively secreted into the media was predominantly (95%) prorenin. However, active renin secretion was stimulated 2.3- and 4.6-fold by treatment with 8-bromo-cAMP after 4 and 15 h, respectively. In addition, the presence of multiple secretory granules was confirmed by ultrastructural analysis. These cells, which express renin mRNA and can regulate secretion of active renin, should provide an excellent tool for studying renin gene regulation and secretion. Furthermore, these mice should provide a useful source for the establishment of renin-expressing cell lines from a variety of renin-expressing tissues.

Mouse renin genes (Ren-1, Ren-2, and Ren-2) exhibit a complicated pattern of gene-specific expression (1-3). Although all three murine genes share greater than 96% homology, they exhibit overlapping yet distinct tissue-specific expression profiles (2). Transgenic mice containing various Ren-2 transgenes have shown that the cis-acting elements controlling renin expression in various organ sites are closely linked to the renin coding regions (4-7). In addition, a Ren-2 genomic segment has been identified which confers a strain-specific, estrus cycle-dependent pattern on adrenal renin expression (6). However, the detailed analysis of a large number of genomic constructs by transgenic mice may be impractical because the number of constructs that can be efficiently examined is limited.

Transfection of mouse and human renin genes into L cells, Chinese hamster ovary cells, and AtT-20 cells has permitted isolation of cell lines which contained immunoreactive renin (8, 9). Furthermore, data suggesting the presence of negative and positive cis-acting regulatory elements in the mouse renin genes transfected into noncognate cells has been reported, although some of these data are conflicting (10-13). However, such noncognate cells do not express their endogenous renin gene and most likely do not contain the correct complement of trans-acting factors required for gene-specific or tissue-specific expression. In addition, although the isolation of renin-expressing cell lines has been reported, many of these reports contain conflicting data regarding the quantitation of renin mRNA. This has prompted us to investigate the possibility of isolating tissue culture cells from various organ sources that express their endogenous renin gene.

We undertook an approach using transgenic mice to direct the expression of a nuclear oncogene (SV40 T antigen) specifically to renin-expressing cells. This was accomplished by fusing 4.6 kb of the 5' flanking sequence from the Ren-2 gene to the T antigen structural gene. T antigen has the capacity to induce a neoplastic phenotype in certain cell types when expression is directed by certain tissue-specific promoters (14-16). Accordingly, we reasoned that it might be possible to isolate renin-expressing tumors and tumor-derived cell lines if T antigen expression was directed to renin-expressing cells. Such an approach has been successful in the isolation of insulin-secreting pancreatic β-cell lines when T antigen expression was driven by an insulin promoter (17). Transfection of SV40 viral DNA into JG cells isolated from a human JG cell tumor has been reported to yield an immortalized human renin-producing JG cell line, although the levels of renin secreted by these cells decreased over time in culture (18, 19). In addition, renin-expressing cells have been reported from a testicular Leydig cell tumor (20, 21).

* This work was funded by National Institutes of Health Grants HL35792, GM30248, GM24125, and CA18420. This project was also supported in part by Biomedical Research Support Grant Grant 807 RR-05648-23 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of National Institutes of Health Fellowship HL07963.

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1 The abbreviations used are: kb, kilobase(s); JG, juxtaglomerular; PBS, phosphate-buffered saline; DMEM, Dulbecco’s minimal Eagle’s media; FBS, fetal bovine serum.
We have previously reported that the 4.6-kb 5' flanking Ren-2 sequence employed in this construct conveys the appropriate tissue- and cell-specific expression profile characteristic of the mouse renin genes throughout fetal development and in adults (22, 23). Here we report the isolation and characterization of nonclonal and clonal renin-expressing cell lines isolated from a primary kidney tumor and ascites.

**EXPERIMENTAL PROCEDURES**

**Production of Transgenic Mice**—The renin T antigen fusion gene, pReg·TAG (Fig. 1), consists of 4.6 kb of mouse Ren-2 promoter (amplification temperature 37 °C) for optimal trypsin activation using trypsin-Sepharose were performed on mouse tissues, following which the 1-h time point and 4 °C temperature were selected (data not shown). Cultured cells and solid tumor samples were normalized for protein content by the method of Bradford (27). Regulated secretion of active renin was assayed by treating cells with 8-BrCAMP for 4 or 10 h as previously described (8).

In order to rule out inadvertent activation of prorenin during the homogenization (which takes place in the absence of protease inhibitors), a pilot experiment was performed in which kidneys and cell lines were homogenized with and without protease inhibitors. There was no significant difference between the active renin content of homogenates made by either method (145.7 ± 28.8 versus 140.2 ± 24.0, not significant for cell lines; 3683 ± 1009 versus 4583 ± 1451, not significant for mouse kidney; expressed as nanograms of A-I/h/mg of protein, mean ± S.D.). These results strongly show that the renin detected without trypsin activation is predominantly active renin and not activated prorenin.

**Ultrastructural Analysis**—For electron microscopy, clone 4.1 cells were grown until 50% confluency in styrene dishes. The cells were fixed in 3% glutaraldehyde, 0.1% tannic acid at 25 °C after washing twice with 37°C Sorensen's buffer, pH 7.2 (0.2 M sodium phosphate). They were postfixed in 2% Oso4 for 15 min at 4°C and rinsed in buffer before dehydration and Epon embedding as described previously (28). Cells were sectioned and visualized after uranyl acetate staining by standard methods.

**RESULTS**

**Characteristics of the ren-T antigen fusion gene**—Transgenic mice were constructed with a fusion transgene consisting of a 4.6 kb of the 5' flanking region of Ren-2d and the large T antigen gene from SV40 in an effort to initiate neoplastic transformation of renin-expressing cells (Fig. 1). In all, eight founder mice were identified by Southern blot analysis of tail DNA (data not shown), three of which either did not breed or have not developed any overt tumors. Transgenic mice from the other transgenic lines (numbers T3, T4, T6, T7, and T9) gave rise to tumors with an onset between 4 and 8 months of age. These animals exhibited tumors involving the kidney, subcutaneous tissue, adrenal gland, and testes (Table I). The occurrence of tumors in the mice varied from line to line as exemplified by the substantially higher frequency of subcutaneous tumors in the T4 transgenic line and the apparent increase in frequency of intraparenchymal kidney tumors in the T7 transgenic line. Nonetheless, each line exhibited...
between these lines is not necessarily surprising considering essentially the same spectrum of tumor types. The difference between these lines is not necessarily surprising considering the stochastic nature of tumorigenesis. Although transgene position effects may cause a difference in the tumor frequency among lines, we feel that differences in the level of expression at the various sites among lines is a more likely explanation. Furthermore, we have previously reported qualitatively correct tissue- and cell-specific expression of this transgene (22, 23).

**Kidney Tumor Histology**—Although kidney tumors involving the renal parenchyma were the most abundant type of kidney tumor, others involving the kidney capsule and peri-renal fat were also seen. The latter generally developed around 4 months of age while the intraparenchymal tumors, all but one of which were unilateral, generally did not develop until 5-8 months of age. The intraparenchymal tumors were usually large, measuring up to a few centimeters in their maximum dimension, often completely replacing the normal renal parenchyma. They presented mixed histological patterns ranging from adenocarcinomas to spindle cell sarcomas with some intermediate or transitory forms. The adenocarcinomas were composed mostly of relatively small glandular structures lined by plump low columnar to cuboidal epithelial cells with pleomorphic and hyperchromatic nuclei, and the stroma contained some small spindle cells (Fig. 2B). The intermediate form frequently assumed an angiosarcomatous pattern with nests and cords of spindle and polygonal tumor cells outlined by an irregular network of vascular channels (Fig. 2, A and C). The sarcomas were composed of loosely arranged neoplastic spindle cells with scattered multinucleated tumor giant cells (Fig. 2, D and E), presenting a fibrosarcomatous pattern. Thus, the general histological appearance of the kidney tumors suggests that they originated in nephroblastic tissue or from vascular smooth muscle. Support for the latter stems from the observation that adult mice that did not show evidence of overt renal tumors often demonstrated marked renal vascular smooth muscle cell hyperplasia (data not shown).

In addition to a renal tumor, the T7-1-6 mouse developed an ascites which probably originated from release of tumor and blood cells from the primary kidney tumor. Histological analysis of this fluid revealed atypical tumoral cells, red blood cells, and lymphocytes. RNA isolated from the ascites cells revealed a low level of renin and transgene mRNAs (data not shown).

**Tumor Renin Expression**—Renin and T antigen expression were next assayed to determine whether tumorigenesis was the direct result of T antigen expression in a renin-expressing cell. As shown in Fig. 3, expression of the endogenous Ren-1<sup>e</sup> gene and the transgene was evident in the primary tumor (1<sup>o</sup>) and in tumors propagated in athymic nude mice (Nu-1). In these mice, the Ren-1<sup>e</sup> gene is the naturally occurring renin allele and encodes the circulating renin. Renin mRNA-containing cells were easily detected in tumor sections by in situ hybridization although the levels of renin message per cell were highly variable (data not shown). In addition, we examined two other kidney tumors from T7 mice, one tumor from a T3 mouse, and bilateral tumors from the T6 founder, and all of them were found to contain renin mRNA.

Renin activity was detected in extracts of the primary tumors and tumors grown in nude mice (Fig. 4A). Since little additional activity was detected after limited proteolysis by trypsin, a procedure which has been shown to activate pro-renin efficiently (25, 26), we suggest that the primary species of renin stored by these tumor samples was active renin. Similarly, active renin was the predominant form of renin found in an independent primary kidney tumor from a different T7 mouse.

**Isolation and Characterization of Cell Lines**—In order to isolate renin-expressing cells in vitro, both lobes of the primary kidney tumor from T7-1-6 were first propagated in nude mice and then were screened for renin expression. The resultant tumors and the primary ascites cells were then seeded into culture dishes. In most cases, the dissociated tumor cells quickly attached to the culture flasks, although it took several months to establish them as continuously growing cell lines. The origin of each cell line is described in Table II. When sufficient numbers of cells were harvested, Northern blots (Fig. 3) and T antigen immunocytochemistry were performed. The results showed that all the culture lines expressed the endogenous renin gene mRNA (Fig. 3A, TC1-5) at levels as great as 25% of the primary tumors (Fig. 3B). The size of the

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**Table 1: Incidence of tumors in transgenic mice**

| Transgenic line | Number of transgenic mice examined | Number of mice examined with tumors | Number of mice perirenal tumors | Number of mice intraparenchymal kidney tumors | Number of mice subcutaneous tumors | Number of mice other tumors |
|-----------------|----------------------------------|-----------------------------------|---------------------------------|---------------------------------------------|-----------------------------------|---------------------------|
| T3              | 3                                | 2                                 | 0                               | 1                                           | 1                                 | 1                         |
| T4              | 23                               | 11                                | 3                               | 0                                           | 11                                | 3                         |
| T6              | 1                                | 1                                 | 0                               | 1                                           | 0                                 | 0                         |
| T7              | 46                               | 19                                | 1                               | 9                                           | 5                                 | 5                         |
| T8              | 1                                | 1                                 | 1                               | 0                                           | 1                                 | 0                         |

**Fig. 2. Kidney tumor histopathology.** A, gross photograph of the kidney tumor from the T6 founder with a small adenocarcinoma in the upper pole. B, photomicrograph of a tubular carcinoma from a T7 offspring. C, photomicrograph of a highly vascular carcinosarcoma of the kidney, from propagation of the tumor shown in B in nude mice. D, higher magnification of C. E, spindle cell sarcoma from a T7 offspring. The bar in B is 40 μm and the bar in C is 0.1 mm. Magnifications of D and E are the same as B.
transgene mRNA (Fig. 3C) and the endogenous renin gene mRNA (Fig. 3A) was consistent with proper processing and transcription initiation. Although some cell lines (TC-1) maintained a constant level of renin mRNA, there was great variability in renin mRNA levels in subcultures from some cell lines recovered from frozen stocks. In addition, there was a low percentage of non-T antigen-containing cells within some of the cultures when examined immunocytochemically, and in situ hybridization revealed fluctuations in renin mRNA across the hybridization slide. Nevertheless, some sections of the slide, most likely containing clonal populations, had equal hybridization intensity among cells.

The above observations, and the fact that the level of renin expression in these nonclonal cultures decreased over long periods of continuous culture, prompted us to isolate clonal cell lines. Single-well isolated colonies from TC-2 were picked and allowed to grow in culture. One of these colonies (clone 4.1) expressed renin mRNA at a level approximately 10-fold greater than the original nonclonal line and maintained this level, with only minor variability among culture flasks, for over 3 months of continuous culture (Fig. 3D). Fig. 5 illustrates the cellular morphology and nuclear localization of immunoreactive T antigen. Since they uniformly contained T antigen (Fig. 5B) and maintained high level expression of renin mRNA after long term continuous culture, we have considered this line homogeneous.

Renin Synthesis in Tumor-derived Cell Lines—The renin activity measured in the cell extracts and media of the nonclonal cells is presented in Fig. 4, B and C. Although the amount of renin was highly variable between cell lines, the culture cells, like the tumors, appeared to store active renin with very little prorenin (Fig. 4B). In contrast, the level of prorenin in the media exceeded that of active renin by more than 10-fold (Fig. 4C). These results suggest that under the conditions described herein, without stimulating the regulated pathway, the cells constitutively secrete prorenin. These experiments were repeated with clone 4.1. In this set of experiments, the cells contained an approximate equal proportion of renin and prorenin (S.E. is ±10%). However, similar to the nonclonal cultures, 95% of the renin in the media was prorenin consistent with the notion that the cells constitutively secrete prorenin. The reason for the difference in intracellular renin storage between the clonal and nonclonal cell lines is unclear; however, variations in the level of

Fig. 3. Renin and T antigen expression in tumors and cell lines. A, 20 μg of liver RNA (LIV), 1.0 and 0.1 μg of DBA/2J submandibular gland RNA (DBA SMG), and 20 μg of tumors and cell lines were hybridized to an antisense renin RNA probe. t'A, one lobe of the primary tumor from T7-1-6; Nu-1, primary tumor propagated in nude mice; TC, tissue culture. TC-1 and TC-3 are TC-1 cells prior to and after rescuing from frozen stocks, respectively. B, the levels of renin mRNA in TC-2 (20 μg) are compared with the primary tumor from which they were derived (20, 5, and 1 μg). C, 20 and 5 μg of the T7-1-6 primary tumor (t'A) and 20 μg of TC-2 and TC-3 were hybridized to an antisense T antigen probe. The characteristic double-banding pattern is seen in all lanes indicating utilization of both the large and small T antigen splice sites. D, dot hybridization for renin mRNA of the clone 4.1 cell line and Ltk- cells (L). Dilutions of DBA/2J submandibular gland RNA is added for comparison (1). The amount of RNA in micrograms is indicated along the sides, and the times (weeks) of continuous culture are along the top.

Fig. 4. Renin activity in tumors, cells, and media. A, the renin activity in the tumor cells is shown. t'A and t'B are two different lobes of the multilobed primary tumor. Nu-1 and -2 are pieces of t'A and t'B, respectively, that were passaged in nude mice. B, renin activity in culture cells. C, renin activity in culture media containing approximately 10⁵ cells over a 72-h period. L41 cells are L cells transfected with a human renin gene constitutively secrete prorenin and were therefore used as a control for trypsin activation of prorenin (8). N is shown above each bar. White bars, active renin; black bars, total renin; stippled bars, prorenin. Trypsin digestion of the inactive renin often results in partial degradation of active renin and the appearance that active renin is greater than total renin activity.
Renin-expressing Cell Lines from Transgenic Mice

FIG. 5. Morphology and T antigen expression in the 4.1 cells. A, hematoxylin- and eosin-stained cells grown directly on glass slides. B, immunocytochemical analysis to identify T antigen-containing cells. C, higher magnification of B. D, antibody reaction omitting the primary antibody. No nuclear staining is evident. No staining was seen in STO or Ltk" cells. The bar in D represents 25 \( \mu \)m for A, C, and D, and 100 \( \mu \)m for B.

Table II
Origin of the cell lines

| Cell line | Origin | Comment |
|-----------|--------|---------|
| TC-1 Nu-1 | Primary (A) kidney tumor propagated in nude mice | |
| TC-2 Asctes | Probably primary tumor cells | |
| TC-3 Asctes | Probably primary tumor cells | |
| TC-4 Nu-1 | Secondary passage Nu-1 in nude mice | |
| TC-5 Nu-2 | Primary (B) kidney tumor propagated in nude mice | |
| Clone 4.1 TC-2 | Cloned colony, homogeneous cell population | |

FIG. 6. Regulated secretion in the 4.1 cell line. The percent active renin secreted into the media in the absence of 10 \( \mu \)M 8-Br-cAMP (DMEM) or in its presence for 4 and 15 h are shown. Inset, dot-blot hybridization for renin mRNA in the absence (–) or present (+) of 8-Br-cAMP. The amount of RNA in micrograms is shown on the side.

In order to determine if these cells could regulate their active renin secretion, they were treated with 8-bromo-cAMP, a known secretagogue of active renin (8). As shown in Fig. 6, active renin secretion was stimulated 2.3-fold \( (p < 0.005) \) after 4 h, and 4.6-fold \( (p < 0.001) \) after 15 h of cAMP treatment. This induction was similar to that previously reported for secretion of renin in transfected A1T-20 cells, cells which contain both constitutive and regulated secretory pathways (8). Renin mRNA transcription was stimulated approximately 3-fold after 15 h of treatment (Fig. 6, inset) possibly due to depletion of intracellular stores of renin.

Ultrastructural analysis (Fig. 7) of clone 4.1 cells revealed the presence of numerous secretory granules morphologically similar to secretory granules of renin-secreting cells of the kidney (29, 30). These results, when taken together, strongly suggest that these cells can regulate active renin secretion.

DISCUSSION

Tumorigenesis in the Renin-T Antigen Transgenic Mice—We set out to isolate renin-expressing cell lines derived from renal and extrarenal sites of renin synthesis with the aim of developing a library of cell lines that could be used to study differential regulation of the mouse renin genes at the various organ sites. So far, we have succeeded in isolating renin-expressing cell lines derived from the kidney. Renin expression in several human JG cell tumors, in congenital mesoblastic nephroma and in certain Wilms' tumors, has been described previously (31-34). To our knowledge, only a few cell lines have been previously reported to express their endogenous renin mRNA including human chorionic cells, human tumoral JG cells, and mouse Leydig tumor cells (18-21, 35). However, a detailed analysis of renin secretion from some of these lines has been hindered because the level of intracellular renin and renin released from them decreased after long term continuous culture.

Although the histopathology of the kidney tumors was consistent with a nephroblastic origin, the positive identification of the cell types which gave rise to them is uncertain. A microscopic examination of nontumorous kidneys from at least two transgenic lines revealed marked hyperplasia in the renal vasculature affecting smooth muscle cells and mesangial cells (data not shown). The site of this pathology is consistent with the pattern of renin expression. For instance, renin expression has previously been shown to occur throughout the renal arterial tree during fetal development (22), and cells further up in the arterial tree from JG cells can be recruited to express renin under certain conditions in adult rodents (36, 37). In addition, renin synthesis has been reported in cultured glomerular mesangial cells (38). These cells may have undergone the secondary events necessary to transform them to a tumorigenic phenotype. That the cell lines regulate active

FIG. 7. Ultrastructure of clone 4.1 cells. Electron microscopy of a representative 4.1 cell. Numerous rounded electron-dense granules are evident at one pole of the cell. Original magnification, \( \times 5000 \).
Renin-expressing and Renin-secreting Cell Lines—We have thus far established and characterized 5 nonclonal cell lines and 1 clonal cell line originating from a kidney tumor and ascites. We are currently establishing other cell lines derived from independent kidney and subcutaneous tumors. We have estimated the renin mRNA content of the 4.1 cell line to be on the order of 1000-2000 copies per cell as estimated by comparing the relative intensity of the Northern Blot hybridization signal to tissues with known renin mRNA content (1). This value is approximately 5-fold lower than the estimated renin mRNA content of JG cells previously reported (1). However, since the level of JG renin expression in a normal kidney can vary substantially depending on the animal’s physiological status, it is difficult to accurately estimate the level of renin expression in these cells prior to the onset of tumorigenesis or during overt tumor formation.

In our cell lines, the predominant form of renin secreted into the media was prorenin (95%). This seems to indicate that the cells utilize the constitutive pathway for prorenin secretion. Similar findings have been reported for mouse cells transfected with a human renin gene (8), for cultured JG cells (19), human choriocarcinoma cells in culture (35), and for isolated submandibular gland and kidney sections (39, 42). Furthermore, a large percentage of the renin found in the plasma of mice is prorenin (43).

Intracellularly, the nonclonal cell lines and primary tumors contained exclusively active renin. The clonal line 4.1, on the other hand, contained an equal mixture of active and inactive renin. It has previously been reported that cultured human JG cells and choriocarcinoma cells generally store less than 3% of the total renin secreted over a 24-h period (19, 35). Although our assay procedures make a direct comparison difficult, our results clearly indicate that there was significant intracellular active renin and stimulation of active renin secretion in response to secretagogue. Moreover, the presence of dense core secretory granules in these cells strongly suggests they are capable of intracellular storage and secretion, although the identification of renin inside these granules will require immuno-electron microscopy.

In conclusion, detailed analysis of renin expression and biosynthesis in vivo has been hindered by the fractional percent JG cells constitute within the intact kidney. We have utilized a system of targeted neoplasia to isolate renin-expressing cell lines from the kidney in vitro. That the cells are capable of regulating secretion of active renin suggests they should offer a useful tool for a detailed examination of its secretory pathways. In addition, they may present an important source of trans-acting factors that interact with renin regulatory sequences, and as such could prove useful for a detailed analysis of the sequences which regulate murine renin gene expression. Furthermore, these mice provide a reproducible source of renin-expressing kidney tumors as well as adrenal, testicular, subcutaneous, and submandibular gland neoplasias. The isolation of renin-expressing cell lines from renal and extrarenal tissues and with different renin gene specificities are feasible by this approach and studies toward this end are in progress.

Acknowledgments—We are grateful to Loren Field for his generous gift of rabbit anti-βT antigen antibody and to Shimon Efrat for his gift of pT3T7-TAG. We would like to thank Frank Pacholec for injecting and implanting eggs and Norifumi Nakamura for performing renin activity assays. We are indebted to Mary K. Ellsworth, Boutros Bouyounes, Alfred Cairo, and Ruth Weaver for their excellent technical assistance. In addition, we would like to thank William Held, John Fabian, Kenneth Abel, and Rita Sigmund for their comments and suggestions regarding the manuscript.

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*J. Biol. Chem.* 1990, 265:19916-19922.

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