Because GK1.5 effectively ameliorates various autoimmune syndromes, we administered the antibody in an identical manner to a control group of mice made diabetic by a large bolus of streptozotocin (but not receiving transplants) to investigate the possibility that we were treating an autoimmune component of streptozotocin-induced diabetes (*17–19*). None of the animals in this group showed signs of recovery (Fig. 2).

Earlier studies had shown that long-term stable endocrine allografts could be rejected if large numbers of splenocytes syngeneic to the allograft were administered to the recipient (*24*). Thus recipient B6 mice that had maintained their allografts for more than 150 days after GK1.5 treatment were challenged with donor-specific allogeneic A/J spleen cells in an attempt to cause rejection of the allograft. The dose of spleen cells used to cause rejection was titrated and, as demonstrated in Fig. 3, "tolerance" could be broken, and long-standing islet grafts were rejected in four of five animals when 5×10^7 A/J spleen cells were administered intraperitoneally. Thus, continued long-term unresponsiveness is not due to the deletion of antigen-reactive effector lymphocytes or to the lack of target alloantigens on the islet grafts.

To further understand the mechanism (or mechanisms) by which treatment with antibody to L3T4 allowed the long-term survivors to maintain their allografts, we considered the possibility that a persistent "hole in the T cell repertoire" after regeneration of the T helper subpopulation might result in a subsequent inability to recognize the alloantigens on the islet allografts. Lymph node cells of naïve mice contain a large proportion of precursor T cells capable of responding to MHC alloantigens (approximately 1:20) (*27*). Therefore, if holes in the T helper repertoire existed, syngeneic B6 lymph node cells from naïve recipients should provide replacement for allorecognition of the "tolerated" A/J allografts. When long-term allograft survivors were given intravenous injections of 10^5 to 10^7 syngeneic (B6) lymph node lymphocytes from naïve recipients, there was no ensuing graft rejection.

Preliminary adoptive transfer studies suggest that active suppression may be responsible for the long-term allograft acceptance. However, the underlying mechanism of graft acceptance is not established. These experiments demonstrate that transient removal of the helper-inducer T cell subset can lead to indefinite allograft survival and underscore the importance of this subset during tissue rejection. Such studies suggest a rationale for similar tolerance induction studies in primates and may lead to the application of this mode of immunotherapy in man.

REFERENCES AND NOTES

1. B. M. Hall and S. E. Dorsch, *Immunol. Rev.* 77, 31 (1984).
2. T. B. Strom, J. W. Jupler-Weglinski, C. D. Heideck, N. L. Tilney, *Transplant. Proc.* 17, 801 (1985).
3. D. W. Mason and P. J. Morris, *Annu. Rev. Immunol.* 4, 119 (1986).
4. J. F. A. P. Miller, *Proc. R. Soc. London Ser. B* **156**, 415 (1962).
5. M. J. Dallman, D. W. Mason, M. Webb, *Eur. J. Immunol.* 12, 511 (1982).
6. A. E. Chang and P. H. Sugarbaker, *Transplantation* 29, 381 (1980).
7. K. J. Lafferty, *Transplant. Proc.* 17, 1549 (1985).
8. S. J. Burakoff, *ibid.*, p. 1546.
9. D. Steinmuller, *Transplantation* 40, 229 (1985).
10. P. Marrack, *J. Exp. Med.* 158, 1077 (1983).
11. D. P. Dialynas, *Immunol. Rev.* 74, 29 (1983).
12. A. M. Krensky and C. Clayberger, *Transplantation* 39, 339 (1985).
13. R. Benjamin and H. Waldmann, *Nature (London)* 320, 449 (1986).
14. R. Benjamin, S. P. Cobbold, M. R. Clark, H. Waldmann, *J. Exp. Med.* 163, 1539 (1986).
15. J. Goronzy *et al.*, *ibid.* **164**, 911 (1986).
16. D. Wofsy, D. C. Mayes, J. Woodcock, W. E. Seaman, *J. Immunol.* 135, 1698 (1985).
17. M. K. Waldor *et al.*, *Science* 227, 415 (1985).
18. D. Wofsy and W. E. Seaman, *J. Exp. Med.* **161**, 378 (1985).
19. G. E. Ranges, S. Sriram, S. M. Cooper, *ibid.* **162**, 1105 (1985).
20. S. P. Cobbold, A. Jayasuriya, A. Nash, T. D. Prospero, H. Waldmann, *Nature (London)* **312**, 548 (1984).
21. G. Brosky and J. Logothetopoulos, *Diabetes* **18**, 606 (1969).
22. P. E. Lacy and M. Kostianovsky, *ibid.* **16**, 35 (1967).
23. P. E. Lacy, J. M. Davie, E. H. Finke, *ibid.* **30**, 285 (1981).
24. D. Faustman, V. Hauptfeld, P. Lacy, J. Davie, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5156 (1981).
25. A. A. Like and A. A. Rossini, *Science* **193**, 415 (1976).
26. Y. T. Kim and C. Steinberg, *Diabetes* **33**, 771 (1984).
27. M. Simonsen, *Cold Spring Harbor Symp. Quant. Biol.* **32**, 317 (1967).

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Tissue Distribution and Developmental Expression of the Messenger RNA Encoding Angiogenin

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New blood vessel growth occurs during normal fetal development and in diseases such as cancer and diabetes. The polypeptide angiogenin induces new blood vessel growth in two biological assays and may play a role in the vascular development of the fetus and in the neovascularization that accompanies diseases and wound healing. A complementary DNA probe for human angiogenin was used to examine the tissue distribution of angiogenin messenger RNA (mRNA) in the developing rat and in selected transformed cell lines. Angiogenin mRNA was detected predominantly in adult liver but was also detectable at low levels in other tissues. The expression of the angiogenin gene in rat liver was found to be developmentally regulated; mRNA levels were low in the developing fetus, increased in the neonate, and maximal in the adult. The amount of angiogenin mRNA in human HT-29 colon carcinoma and SK-HEP hepatoma cells was not greater than that in normal rat liver. These results demonstrate that angiogenin is predominantly expressed in adult liver, that the pattern of angiogenin gene expression is not temporally related to vascular development in the rat, and that the transformed cells studied do not contain more angiogenin mRNA than does normal liver. If angiogenin activity is controlled at the transcriptional level, the results of this study suggest that the primary function of angiogenin in vivo may be in processes other than the regulation of vascular growth.

ANGIOGENIN, A POLYPEPTIDE WITH a molecular size of 14 kD, was originally isolated from conditioned media of the HT-29 human adenocarcinoma cell line (*1*). Angiogenin is an inducer of vascular growth in the chick chorioallantoic membrane and the rabbit cornea and has been suggested to be a mediator of vascular development (*2*). At the amino acid level, angiogenin has significant homology with pancreatic ribonuclease and catalyzes the cleavage of 28*S* and 18*S* ribosomal RNA (*3*). The significance of the ribonuclease activity of angiogenin is unclear. Angiogenin contains a signal peptide characteristic of secreted proteins, but the site of synthesis, the stimulus for secretion, and the site of action of angiogenin are unknown. The human angiogenin gene has been cloned and sequenced (*4*), but the tissue distribution and developmental regulation of angiogenin gene expression have not been described. This report details an analysis of

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angiogenin gene expression, as assessed by messenger RNA (mRNA) levels, in the developing rat and in selected transformed human cell lines.

As the original complementary DNA (cDNA) clone for angiogenin was not available, we cloned the cDNA from a λgt11 adult human liver cDNA library (Clontech Laboratories, Palo Alto, California) using oligonucleotide probes complementary to the published angiogenin nucleotide sequence (*4*). Primary screening of 300,000 recombinants with two 99-nucleotide probes complementary to the nucleotides encoding amino acids 30 to 62 and 92 to 123 of human angiogenin yielded 26 recombinants that strongly hybridized to both probes. The five recombinants that were further evaluated also hybridized to a third oligonucleotide complementary to the angiogenin signal peptide sequence. These recombinants were purified and each contained an insert of 650 to 700 bp. One 700-bp insert was subcloned into the plasmid vector pBSM13$^+$ (Stratagene, San Diego, California), and an Eco RI fragment containing the insert was isolated for use in the expression studies. Double-stranded sequencing (*5*) with forward and reverse M13 primers as well as oligonucleotide primers complementary to the coding region of the cDNA confirmed that the insert encodes human angiogenin.

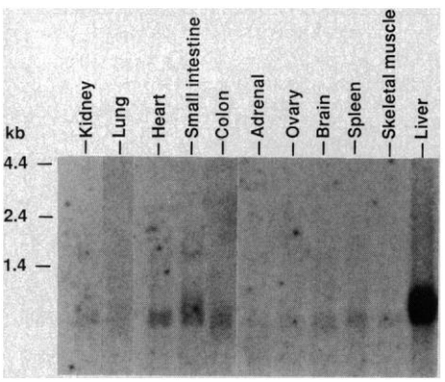

Fig. 1. The tissue distribution of mRNA encoding angiogenin was examined in the rat. RNA from adult rat organs was isolated by the guanidine isothiocyanate–cesium chloride method (*22*), and poly(A)$^+$ RNA was obtained by using oligo(dT) cellulose (*23*). Poly(A)$^+$ RNA (5 to 10 μg) was fractionated on a 1% denaturing agarose gel and transferred to a nylon membrane (Nytran, Schleicher & Schuell, Keene, New Hampshire) in 10× sodium chloride and sodium citrate (SSC). The angiogenin cDNA was labeled with [^{32}P]dCTP by the random hexamer method (*24*), and the filters were hybridized at 42°C for 12 to 20 hours in a solution containing 5× SSC, 50% formamide, 1× Denhardt's solution, 50 m*M* tris (*p*H 7.5), 0.05% sodium pyrophosphate, denatured salmon sperm DNA (0.1 mg/ml), and 1% SDS. Filters were washed twice in 2× SSC, 0.1% SDS, and 0.05% sodium pyrophosphate at room temperature, then twice in 0.1× SSC, 0.1% SDS, and 0.05% sodium pyrophosphate at 60°C, and then exposed to Kodak XAR film for 48 hours with double intensifying screens. The mRNA sizes were determined by comparison with an RNA standard (Bethesda Research Laboratories).

The tissue specificity of angiogenin gene expression was assessed by Northern blot analysis of polyadenylated [poly(A)$^+$] RNA (Fig. 1). In the adult rat, angiogenin is expressed predominantly in the liver as a single poly(A)$^+$ RNA 1 kb in size. Angiogenin is also detectable in poly(A)$^+$ RNA isolated from other tissues, including kidney, lung, heart, small intestine, colon, adrenal, ovary, spleen, and brain. Densitometric analysis demonstrates that the angiogenin mRNA in liver is 20 to 100 times greater than that observed in nonhepatic tissues.

The tissue distribution of angiogenin mRNA in the newborn rat was compared with that in the adult (Fig. 2). Angiogenin is easily detectable in total RNA from liver, but no angiogenin mRNA is detectable in total RNA from nonhepatic tissues of neonatal or adult rat. Furthermore, no angiogenin mRNA is detectable in poly(A)$^+$ RNA from newborn rat brain or heart.

The developmental pattern of angiogenin gene expression in the liver was studied by Northern analysis of poly(A)$^+$ RNA from rat liver at five stages of development (Fig. 3). Angiogenin message is undetectable in the body of the 10-day rat embryo and is expressed at low levels in 15-day and 19-day fetal rat liver. Densitometric analysis revealed that angiogenin mRNA content in the liver increases by a factor of 10 between day 19 of gestation and postnatal day 2. The angiogenin mRNA content increases further during growth to adulthood to twice that found in the neonate.

We have also studied angiogenin gene expression in selected cell lines (Fig. 4). As noted earlier, angiogenin was originally purified from the conditioned media of HT-29 colon carcinoma cells. Angiogenin mRNA is detected in HT-29 cells as a 1.1-kb mRNA. Densitometric analysis suggests that the angiogenin mRNA content of HT-29 cells is approximately twice that of normal rat colon when normalized to α-tubulin. SK-HEP cells, derived from a human hepatoma, also contain angiogenin mRNA in quantities similar to those seen in HT-29 cells. The angiogenin mRNA content of adult rat liver is at least three times that of either HT-29 or SK-HEP cells. Bovine capillary endothelial cells in culture did not contain angiogenin message. Although the human angiogenin cDNA is homologous to the rat angiogenin gene as demonstrated by Northern analysis, it is possible that the bovine gene is not sufficiently homologous to the human gene to be detected. This appears unlikely since even under conditions of low stringency no mRNA could be detected in bovine capillary endothelial cells.

Angiogenesis is a complex process that involves proteolysis of the basement membrane, as well as endothelial cell migration and proliferation (*6*). Several peptides, including angiogenin, acidic fibroblast growth factor (acidic FGF) (*7*), basic fibroblast growth factor (basic FGF) (*8*), and transforming growth factor–α (TGF-α) (*9*), are capable of inducing vascular growth in biologic assays such as the chick chorioallantoic membrane assay (acidic and basic FGF) and the hamster cheek pouch assay (TGF-α). These proteins have been postulated to regulate vascular growth in vivo. However, the known properties of angiogenin differ markedly from those of the other angiogenic growth factors. Basic FGF and acidic FGF are potent endothelial cell mitogens and appear to act through specific membrane receptors (*10, 11*). Similarly, TGF-α binds to the epidermal growth factor receptor and is mitogenic for murine lung microvascular endothelial cells and pulmonary artery endothelial cells in primary culture. Angiogenin has not been reported to be mitogenic for fibroblasts or endothelial cells (*12*), and no receptors for the protein have been described. Our data on angiogenin gene expression further highlight the differences at the mRNA level between angiogenin and other angiogenic polypeptides. Angiogenin mRNA is detectable in all tissues studied but is most abundant in the adult rat liver. The levels of expression in HT-29 adenocarcinoma and SK-HEP hepatoma cells are lower than that of normal rat liver, and no angiogenin mRNA could be detected in bovine capillary endothelial cells. In contrast, basic FGF mRNA is undetectable by Northern analysis in all tissues except bovine hypothalamus, but is present at high levels in bovine capillary endothelial cells and SK-HEP cells (*13*). In addition, while TGF-α

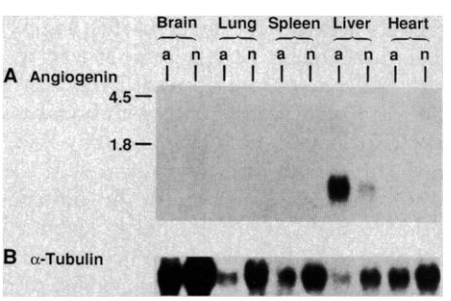

Fig. 2. (**A**) The tissue distribution of angiogenin mRNA in newborn (n) rat was compared to that in the adult (a). Northern blots with 10 μg of total RNA per lane from adult and newborn rat organs were prepared as described in Fig. 1. (**B**) The same filter was washed and rehybridized to a [^{32}P]dCTP-labeled 1.3-kb chicken α-tubulin cDNA. The molecular weights of 28*S* and 18*S* ribosomal RNA are shown at left.

mRNA has been detected at low levels in rat liver, brain, and kidney, expression is 10 to 20 times greater in transformed Fisher rat embryo cells (*14*). Finally, acidic FGF mRNA has to this date been detected only in brain (*15*). Thus angiogenin mRNA appears to be considerably more abundant and widely distributed in tissues than mRNAs encoding other angiogenic proteins.

On the basis of its ability to induce vascular growth in chick chorioallantoic membrane and rabbit cornea, angiogenin has been described as "organogenic" (*2*)—that is, capable of inducing formation and growth of a vascular system in vivo. Thus, the developmental pattern of angiogenin gene expression seen in these studies was unanticipated. In the developing fetus where vascular growth is rapid, and where one would expect increased expression of genes encoding angiogenic polypeptides, angiogenin mRNA levels are low or undetectable. In adult tissues where new vascular growth is slow [as shown by endothelial cell turnover times of approximately 100 to 1000 days in adult mouse organs (*16*)], angiogenin mRNA is detectable in all tissues examined and is particularly abundant in the liver. A markedly different developmental pattern has been described for TGF-α in the rat, where mRNA is abundant in the first week of gestation and declines to undetectable levels by day 13 (*17*). If angiogenin activity is regulated at the transcriptional level, the developmental pattern of angiogenin gene expression in the rat suggests that angiogenin does not mediate vascular growth during fetal development. If angiogenin is an inducer of vascular growth, then its activity in the adult must be tightly regulated at the post-transcriptional level, because angiogenin mRNA is actively expressed in adult tissues in which vascular growth is virtually absent. The recent demonstration that human placental ribonuclease inhibitor binds to angiogenin and abolishes its activity (*18*) suggests that angiogenin may indeed be regulated, at least in part, at the post-transcriptional level. Post-transcriptional control occurs with both basic FGF and acidic FGF (*11, 19*), but mRNA levels are reported to be low in all tissues examined even when the protein content is high. At the present time, neither a specific assay for angiogenin nor an antibody to angiogenin is available. Thus, the possibility that angiogenin activity is regulated at a post-transcriptional level cannot currently be examined.

As noted, angiogenin is predominantly expressed in the adult liver. Since the protein has a signal peptide (*3*), it could be secreted and act in an endocrine fashion on distant tissues. Alternatively, hepatic angiogenin could regulate vascular growth selectively in the liver by paracrine mechanisms. The liver is unique among mammalian organs in its ability to undergo active growth in response to injury (*20*), and the vascular endothelium proliferates rapidly during hepatic regeneration (*21*). Thus, angiogenin could participate in processes primarily involving the liver.

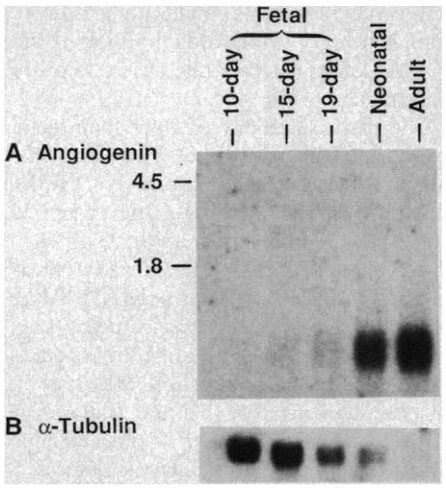

Fig. 3. (**A**) The angiogenin mRNA content of rat liver was examined during development. Poly(A)$^+$ RNA from 10-day rat embryo bodies, 15-day and 19-day fetal rat liver, and neonatal and adult rat liver was isolated as described in Fig. 1. Each mRNA (10 μg) was fractionated on a 1% denaturing agarose gel, transferred, hybridized, and washed as described in Fig. 1. The molecular weight of 28*S* (4.5 kb) and 18*S* (1.8 kb) ribosomal RNA are shown at left. (**B**) The same filter washed and rehybridized to a [^{32}P]dCTP-labeled 1.3-kb chicken α-tubulin cDNA.

Another potential role for angiogenic polypeptides is in the neovascularization of solid tumors. If these proteins stimulate vascular growth in tumors, then one might expect increased angiogenic protein or mRNA content (or both) in transformed cells. For example, basic FGF mRNA is found in greater amounts in SK-HEP cells than in normal tissues (*13*). We do not find a similar relation between angiogenin mRNA content in the transformed cell lines examined and in tissues. Although HT-29 colon carcinoma cells have about twice the angiogenin mRNA content found in normal rat colon, neither HT-29 or SK-HEP cells have as much angiogenin mRNA as normal rat liver. Thus, in contrast to basic FGF, increased angiogenin gene expression is not uniformly detected in the transformed cell lines studied. If angiogenin mediates tumor neovascularization, these results suggest that a post-transcriptional mechanism controls the process.

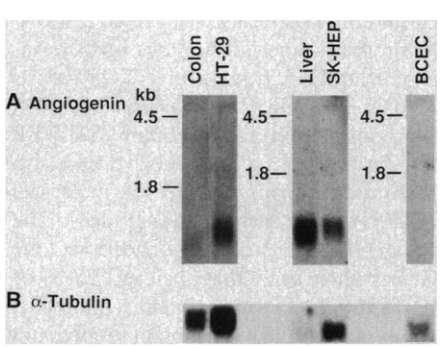

Fig. 4. (**A**) The angiogenin mRNA content of selected cell lines was compared to that in normal tissues. Northern blots of poly(A)$^+$ RNA were prepared and hybridized to angiogenin as described in Fig. 1. (**B**) The same filter washed and rehybridized to chicken α-tubulin. The locations of 28*S* and 18*S* ribosomal RNA are shown at left. BCEC, bovine capillary endothelial cells.

The biological role of angiogenin is still unknown. The data presented in this report show that angiogenin mRNA content in cells is not temporally correlated with vascular growth in the developing rat. If angiogenin is important in regulating vascular growth, complex post-transcriptional mechanisms must exist to regulate angiogenin activity. Until further studies of the in vivo distribution and sites of action of angiogenin protein are reported, the biological role of this protein will remain unclear.

REFERENCES AND NOTES

1. J. W. Fett *et al.*, *Biochemistry* **24**, 5480 (1985).
2. D. J. Strydom *et al.*, *ibid.*, p. 5486.
3. R. Shapiro *et al.*, *ibid.* **25**, 3527 (1986).
4. K. Kurachi, E. W. Davie, D. J. Strydom, J. F. Riordan, B. L. Vallee, *ibid.* **24**, 5494 (1985).
5. E. Y. Chen and P. H. Seeburg, *DNA* **4**, 165 (1985).
6. J. Folkman, in *Important Advances in Oncology*, R. Winters, Ed. (Lippincott, Philadelphia, 1985), pp. 42–62.
7. K. A. Thomas *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6409 (1985).
8. J. A. Abraham *et al.*, *Science* **233**, 545 (1986).
9. A. B. Schreiber *et al.*, *ibid.* **232**, 1250 (1986).
10. D. Gospodarowicz, G. Neufeld, L. Schweigerer, *Mol. Cell. Endocrinol.* **46**, 187 (1986).
11. L. J. Pike *et al.*, *J. Biol. Chem.* **257**, 14628 (1985).
12. L. Liotta, *Nature (London)* **318**, 14 (1985).
13. J. A. Abraham *et al.*, *EMBO J.* **5**, 2523 (1986).
14. D. C. Lee *et al.*, *Nature (London)* **313**, 489 (1985).
15. M. Jaye *et al.*, *Science* **233**, 541 (1986).
16. B. Hobson and J. Denekamp, *Br. J. Cancer* **49**, 405 (1984).
17. D. Lee *et al.*, *Mol. Cell. Biol.* **5**, 3644 (1985).
18. R. Shapiro and B. Vallee, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2238 (1987).
19. T. Maciag, G. Hoover, R. Weinstein, *J. Biol. Chem.* **257**, 5333 (1982).
20. N. Bucher and R. Malt, *Regeneration of Liver and Kidney* (Little, Brown, Boston, 1971), p. 17.
21. J. Widman and H. Fahimi, *Am. J. Pathol.* **80**, 349 (1975).
22. J. M. Chirgwin *et al.*, *Biochemistry* **18**, 5294 (1979).
23. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1982), pp. 193–194.
24. A. P. Feinberg and B. Vogelstein, *Anat. Biochem.* **132**, 6 (1983).

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