Analysis of Insulin-Like Growth Factor I Gene Expression in Malignancy: Evidence for a Paracrine Role in Human Breast Cancer

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Insulin-like growth factor I (IGF-I) activity has been reported to be produced by several human cancers. Identification of RNAs transcribed from the IGF-I gene has been complicated by the detection of multiple hybridizing bands on Northern analysis. To determine if any of these RNAs are transcribed from the IGF-I gene, we have used a sensitive and specific ribonuclease (RNAse) protection assay for IGF-I. We have also studied the breast cancer tissue expression of IGF-I using *in situ* hybridization histochemistry. We have found no IGF-I mRNA in breast (zero of 11) or colon cancer (zero of 9) cell lines; both of these tumors have been previously reported to express IGF-I mRNA. However, three of three neuroepithelioma and one of two Ewing's sarcoma cell lines express IGF-I mRNA; therefore, in these tumors IGF-I may be an autocrine growth factor. In contrast to breast cancer cell lines, RNA extracted from breast tissues has easily detectable IGF-I mRNA. *In situ* hybridizations show that IGF-I mRNA is expressed in the stromal cells, and not by normal or malignant epithelial cells. These findings suggest that although IGF-I is not produced by breast epithelial cells it may function as either a paracrine stimulator of epithelial cells or an autocrine stimulator of stromal cells. (Molecular Endocrinology 3: 509–517, 1989)

INTRODUCTION

As a mediator of the effects of GH, insulin-like growth factor I (IGF-I) plays an important role in normal growth and development (1). IGF-I has also been shown to be a mitogen for cancer cell lines, and several human cancers have been reported to express IGF-I mRNA or protein. Therefore, it has been postulated to function as an important autocrine growth factor in transformed cells (2–8).

Additionally, IGF-I has been shown to be synthesized by normal fibroblasts in culture (9). In the human fetus, *in situ* hybridization studies have localized IGF-I mRNA expression primarily to stromal tissues (10). If IGF-I *in vivo* is produced primarily by stromal tissues, then it could also stimulate the growth of malignant epithelial cells in a paracrine manner.

Previous work has shown that medium conditioned by breast cancer cell lines contains a protein that can be detected by IGF-I RIA. Hybridization of a Northern blot of several poly(A)$^+$-selected breast cancer cell RNAs with an IGF-I cDNA probe detected only one 300 basepair (bp) species that was common to the transcripts detected in adult human liver (2). Larger transcript sizes found in breast cancer cell line mRNA did not correspond to the larger transcript found in liver. This observation has been made in other tumors as well (6, 7). Northern analysis of colon and hepatocellular cancer RNAs using an IGF-I probe revealed multiple hybridizing bands, and it is unclear which, if any, of these bands are transcribed from the IGF-I gene. The various transcript sizes in these cancer cell lines could be explained by differential splicing of the IGF-I gene or hybridization of the IGF-I cDNA probe to closely related RNA species.

To better examine the expression of IGF-I mRNA in cancer cell lines we used an antisense RNA probe transcribed from a 519-bp IGF-IA cDNA (11) in a ribonuclease (RNAse) protection assay. This probe contains exons 1, 2, 3, and 5, which includes the entire coding region of the IGF-IA precursor molecule (12). To localize IGF-I mRNA in breast tissue, we have performed *in situ* hybridization with the same probe. Since all of the coding exons of the IGF-I gene have been cloned, this probe would recognize any mRNA transcribed from the IGF-I gene encoding a similar factor even if the mRNAs included alternate 5′ or 3′ untranslated exons. Using these techniques we have found that none of the breast cancer cell lines we have ex-

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amined expressed the authentic IGF-I gene. We have surveyed several other tumor cell lines and have only found IGF-I gene expression in the related primitive neuroectodermal tumors (PNETs), neuroepithelioma and Ewings' sarcoma. In contrast, IGF-I mRNA was easily found in RNA derived from breast tissue. *In situ* hybridization revealed that the IGF-I mRNA was contained entirely in the stromal tissues of the breast. Since IGF-I gene expression was not found in breast or colon cancer cell lines, authentic IGF-I is unlikely to be an autocrine growth factor for these epithelial tumors. However, IGF-I mRNA expression was found in the stromal elements of the breast, and there IGF-I may be a paracrine growth factor for breast epithelial cells and/or autostimulate stromal components.

RESULTS

IGF-I mRNA Expression by Breast Cancer Cell Lines

Using the RNAse protection assay, IGF-I mRNA was easily detected in 30 μg total RNA obtained from human liver (Fig. 1A, lane 1) and can be seen in as little as 0.5 μg poly(A)$^+$-selected RNA from human liver (Fig. 1A, lane 2). Both IGF-IA and IGF-IB transcript forms can be detected. Since the IGF-IB cDNA cloned from human liver has a different terminal 3′ exon (exon 4) than the IGF-IA cDNA (12), the IGF-IB transcripts will not protect the 3′ end of the IGF-IA probe, and the fragment protected by the IGF-IB transcripts are 99 bp smaller. In contrast, no IGF-I mRNA was found in any (zero of 11) of the human breast cancer cell lines we examined. To maximize the sensitivity of detection, we increased the amount of total cellular RNA hybridized (Fig. 1A, lanes 4 and 6), analyzed only poly(A)$^+$-selected RNA or nuclear RNA, decreased the hybridization temperature, and decreased the exposure time to RNAse A. Despite these maneuvers, no full-length or partially protected transcripts were detected (data not shown). To demonstrate the sensitivity of RNAse protection assays, we have transcribed unlabeled sense RNA from the IGF-IA gene and have determined that this technique can detect less than 1 pg IGF-I transcript (Fig. 2). This correlates with the ability to detect approximately one IGF-I transcript per three cells. Rotwein *et al.* (13) have also demonstrated that RNAse protection analysis is 100-fold more sensitive in detecting IGF-I transcripts than Northern analysis. Therefore, it is unlikely that the immunoreactive IGF-I detected in conditioned medium is translated from mRNA transcribed from the coding exons of the IGF-I gene.

IGF-I mRNA Expression by Other Cancer Cell Lines

IGF-I has also been reported to be an autocrine growth factor in other types of tumor cell lines. Table 1 lists cell lines that we have examined; none expressed IGF-I mRNA. Of note is that IGF-I mRNA was not found in several (zero of nine) colon cancer cell lines, a tumor that has been reported to express IGF-I mRNA (6). However, IGF-I mRNA was found in three of three neuroepithelioma cell lines and one of two Ewing's sarcoma cell lines (Fig. 3). Other related neural tumors, neuroblastoma and glioblastoma, did not express IGF-I mRNA (Table 1 and Fig. 3). Thus, IGF-I could be an autoregulatory factor for neuroepithelioma and Ewing's sarcoma.

IGF-I mRNA Expression by Breast Tissues

We also examined breast cancer tissue samples to see if IGF-I mRNA could be detected *in vivo*. We found that 12 of 20 breast cancer tissue samples contained IGF-I mRNA (Fig. 1B and data not shown), and the levels of IGF-I mRNA vary approximately 10-fold between positive samples. However, Fig. 1C also shows that normal tissue adjacent to the cancers as well as benign mammary fibroadenoma expressed IGF-I mRNA, and the level of expression was quite high compared to that of other benign tissues. Since tissue samples are composed of many different cell types and fibroblasts are known producers of IGF-I protein (9), it seemed possible that the IGF-I mRNA could be derived from the normal stromal elements. In support of this concept, IGF-I mRNA could easily be detected in normal fibroblast cell lines (data not shown).

To further identify the source of IGF-I mRNA in breast cancer, we examined tissue specimens by *in situ* hybridization with the same RNA probe used in the RNAse protection assays labeled with [^{35}S]UTP. The histological section of a breast cancer biopsy shown in Fig. 4A demonstrates an area of malignant tissue as well as an area of surrounding nonmalignant lobules (outlined by *dashed lines*). An autoradiogram of the same section showed that the IGF-I mRNA was contained primarily in the area containing nonmalignant lobules (Fig. 4B). A high powered view of this area revealed that the IGF-I mRNA was localized to the stromal cells (Fig. 4C). Examination of a different biopsy specimen revealed intense IGF-I mRNA hybridization in the stromal cells (Fig. 5, A) of the nonmalignant lobules and not by breast epithelial cells. This pattern of hybridization was also seen in fiboradenoma (Fig. 5B), with IGF-I mRNA contained in the stromal elements of the tumor. Examination of several other tissue specimens confirmed that the IGF-I mRNA was only seen in the stromal cells, and no IGF-I mRNA was detected in the normal or malignant epithelial cells (Paik, S., manuscript in preparation). This hybridization was specific, since no hybridization was seen when a labeled sense IGF-I probe was used (data not shown). Furthermore, *in situ* hybridization did not detect related IGF mRNA species, since no hybridization was seen between the IGF-I probe and the cell lines T47-D or HepG2 (data not shown), both of which express IGF-II mRNA (14). These findings suggest that the IGF-I mRNA detected in breast cancer tissue sample RNAs originated from cells of stromal origin and not from malignant epithelial cells.

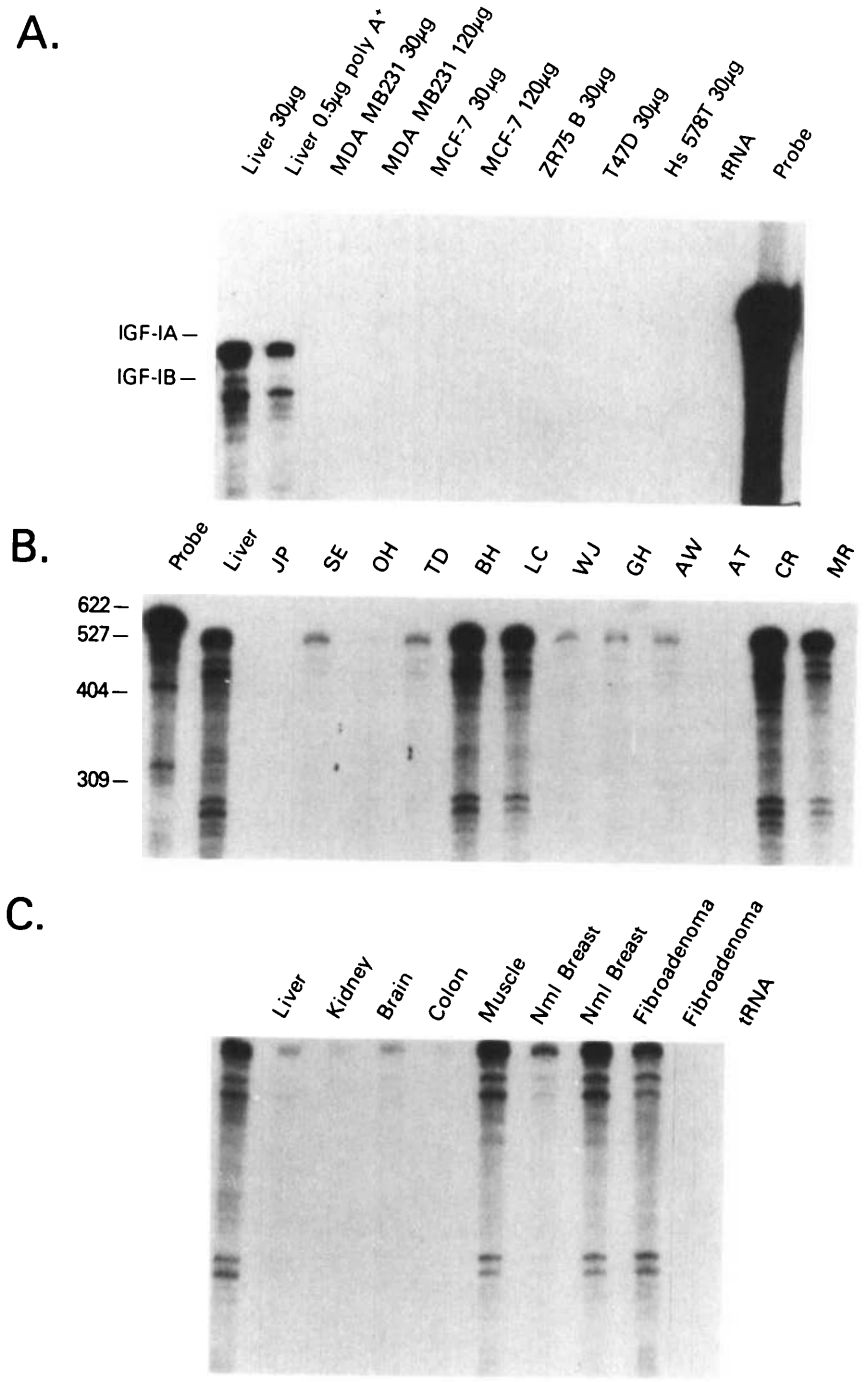

Fig. 1. Expression of IGF-I mRNA in Breast Carcinoma Cell Lines, Breast Carcinoma Tissues, and Normal Tissues

A, RNAse protection assay revealed that IGF-I was not expressed by breast cancer cell lines. IGF-IA and IGF-IB transcripts were easily detected in 30 μg total RNA from human liver (lane 1) or 0.5 μg poly(A)$^+$ RNA from liver (lane 2). Other lanes contained either 30 or 120 μg total cellular RNA extracted from breast cancer cell lines. Autoradiogram exposure was for 48 h. B, IGF-I mRNA expression in 20 μg breast cancer RNA. Each set of initials represents an individual patient. Autoradiogram exposure was for 12 h. C, IGF-I mRNA expression by 20 μg normal tissues and mammary fibroadenoma. Samples labeled Nml Breast were obtained by dissecting grossly normal breast tissues away from breast cancer. Autoradiogram was exposed for 12 h.

DISCUSSION

Although IGF-I transcripts have been reported in human epithelial cancers, the origin of the transcript sizes seen on Northern analysis are not entirely understood. An IGF-I cDNA has been cloned from leiomyosarcoma, a cancer of mesodermal origin (3). In this tumor the transcript pattern seen on Northern analysis was identical to that seen in adult human liver, and it is likely

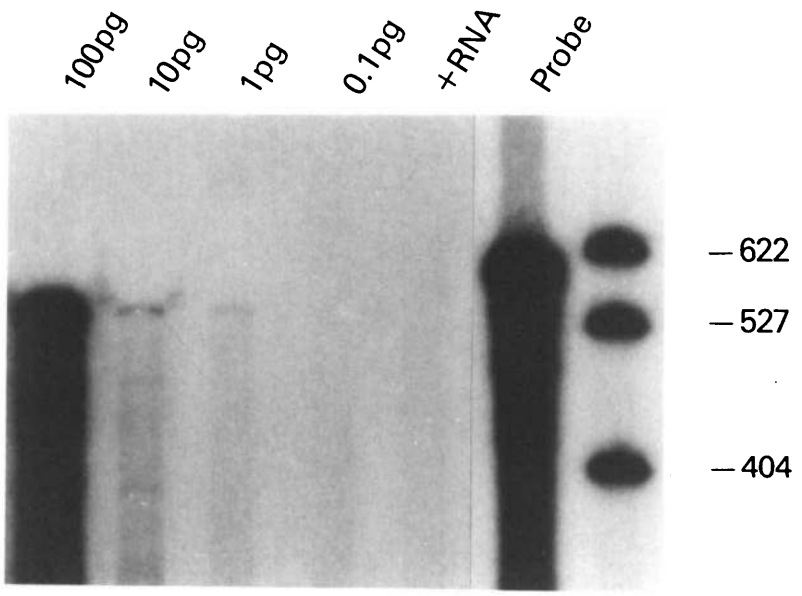

Fig. 2. Determination of the Sensitivity of IGF-I RNAse Protection Assay

Unlabeled sense RNA was synthesized from the IGF-I cDNA, and the concentration was determined by spectrophotometer. One microgram was serially diluted and mixed with 30 μg tRNA. The autoradiogram was exposed for 10 days.

Table 1. Cell Lines Examined for IGF-I mRNA Expression

Cell Lines		
Breast Cancer	Nephroblastoma	Neuroblastoma
MCF-7	SK-Nep 1	Lan 1
T47D	G401	Lan 5
Cama I	Hepatoblastoma	SK-N-SH
MDA-MB 231	HepG2	SY5Y
MDA-MB 468	SK-Hep 1	BE2
MDA-MB 436	Adrenal Cortical Cancer	KCNR
Hs578T	SW13	KAN
BT549	Gastric Cancer	SAN
SKBR3	Kato III	Medulloblastoma
HBL100	Prostate Cancer	TE671
DU4475	LNCap	Glioblastoma
Colon Cancer	DU145	U-373 MG
DLDI	PC3	U-138 MG
LS174T	Squamous Cancer	A172
SW620	A431	Melanoma
Colo 201	Pancreatic Cancer	3711T
Colo 205	Capan 2	3705J
Colo 320DM		
SW948		
Hct 15		
HT29		

None of these cell lines expressed IGF-I mRNA. Twenty or 30 μg RNA was used in an IGF-I ribonuclease protection assay.

that tumors derived from mesodermal precursors express authentic IGF-I. Although it appears that the some untranslated exons of the IGF-I gene have not yet been identified, we have used a probe that covers the entire coding region of IGF-I in a RNAse protection assay and have been unable to identify any IGF-I transcripts protected by this probe in several human malignancies. We conclude that the bands seen on IGF-I Northern analysis of breast cancer RNAs are not transcribed from the identified exons of the IGF-I gene that encode the protein. They either represent related cross-hybridizing RNA species or mRNA transcripts derived from other noncoding exons of the IGF-I gene. Moreover, Northern analysis and dot blot hybridization are inadequate to identify authentic IGF-I transcripts, because these false positive hybridizing species may be detected.

We have found that expression of IGF-I mRNA by epithelial tumors is uncommon; however, tumors of

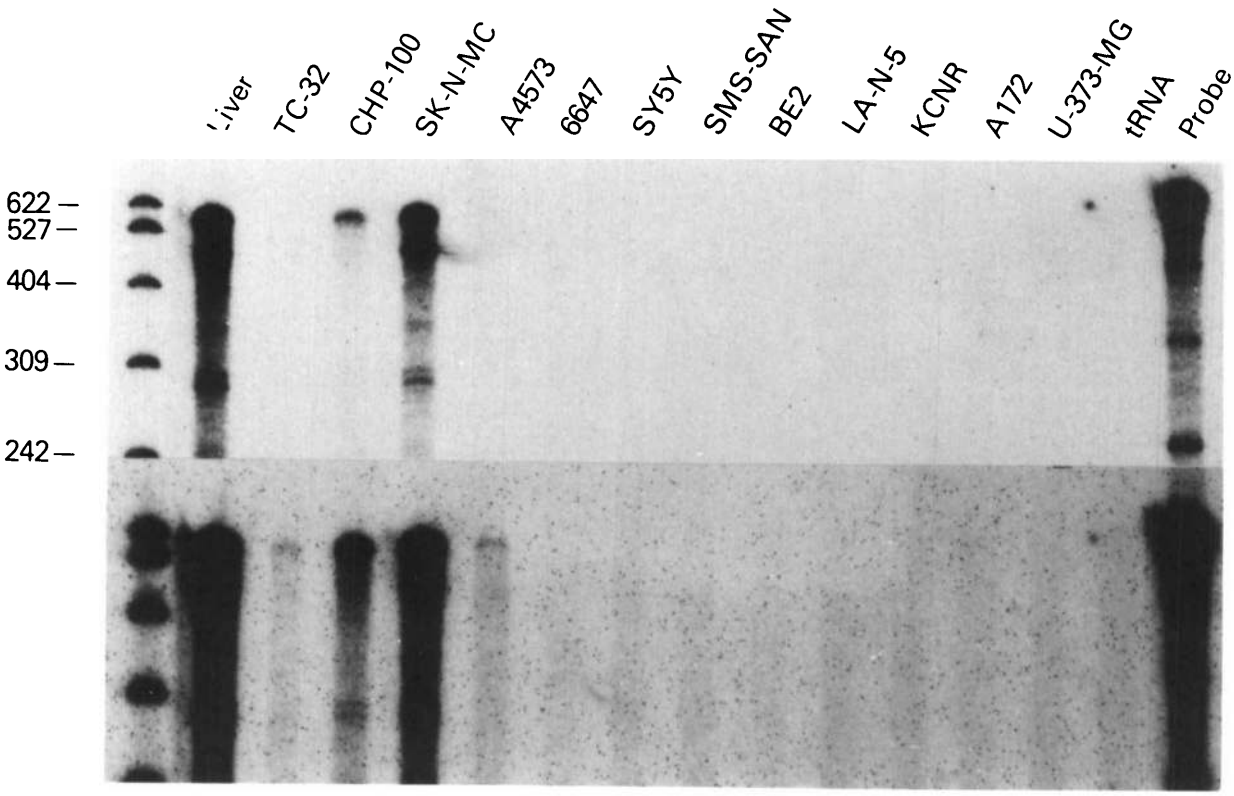

Fig. 3. IGF-I Expression by Tumors of Neural Origin

IGF-I mRNA was detected in 30 μg total cellular RNA from neuroepithelioma cell lines (CHP-100, TC32, and SK-N-MC) and one Ewing's sarcoma cell line (A4573 and not 6647), but not in glioblastoma (A172 and U-373-MG) or neuroblastoma (SY5Y, SMS-SAN, BE2, LA-N-5, and SNS-KCNR). The *upper autoradiogram* was exposed for 12 h; the *lower autoradiogram* was the same gel exposed for 96 h.

neural origins, neuroepithelioma and Ewing's sarcoma, expressed IGF-I mRNA. These tumors along with neuroblastoma are classified as primitive neuroectodermal tumors (15). Both neuroepithelioma and Ewing's sarcoma share a t(11;22) (q24;12) chromosomal translocation, have similar patterns of protooncogene expression (16, 17) and are thought to arise from a common progenitor cell. However, it appears that not all tumors of neural origin have the ability to express IGF-I, since no IGF-I mRNA was found in neuroblastoma. This suggests that IGF-I expression may be specific for a subset of PNETs, and differential IGF expression by neural tumors may be useful as a means of classification. IGF-I mRNA expression has been detected in embryonic cultured rat neurons (18), and unregulated expression of IGF-I by neuronal progenitor cells could lead to autocrine growth stimulation of PNETs. We are currently determining if IGF-I is an autocrine growth factor for neuroepithelioma cells.

The immunoreactive IGF-I protein found in the medium conditioned by breast cancer cell lines either represents an IGF-I-related protein or an IGF-I-binding protein. We and others have demonstrated production of binding protein by these cell lines (19, 20), and it is known that IGF-I-binding proteins can interfere with the RIA for IGF-I (21). However, since the IGF-I cDNA will hybridize to some transcripts on Northern analysis, it is possible that a RNA species with nucleotide homology to IGF-I is transcribed in breast cancer. We are currently working to fully characterize the radioimmunoreactive IGF-I to determine if this protein is related to authentic IGF-I and could account for the transcripts detected on IGF-I Northern analysis of breast cancer cells. This study also demonstrates that to fully identify an immunoreactive IGF-I-related species as authentic IGF-I, either amino acid composition of the protein or RNAse protection analysis of the producer cells with an IGF-I cDNA probe should be performed.

Although breast cancer cell lines expressed no IGF-I mRNA, most breast cancer tissue specimens contained easily detectable IGF-I mRNA. *In situ* hybridizations have shown IGF-I mRNA is expressed by stromal cells of the normal fetus (10). In breast cancer tissue specimens, IGF-I mRNA was also expressed by stromal cells and not by malignant epithelial cells. Since RNA obtained from cancer tissue specimens will also contain RNA derived from both stromal and epithelial components, the cellular origin of any IGF-I mRNA will be ambiguous. Therefore, cancer tissue specimens should be examined by *in situ* hybridization studies before it

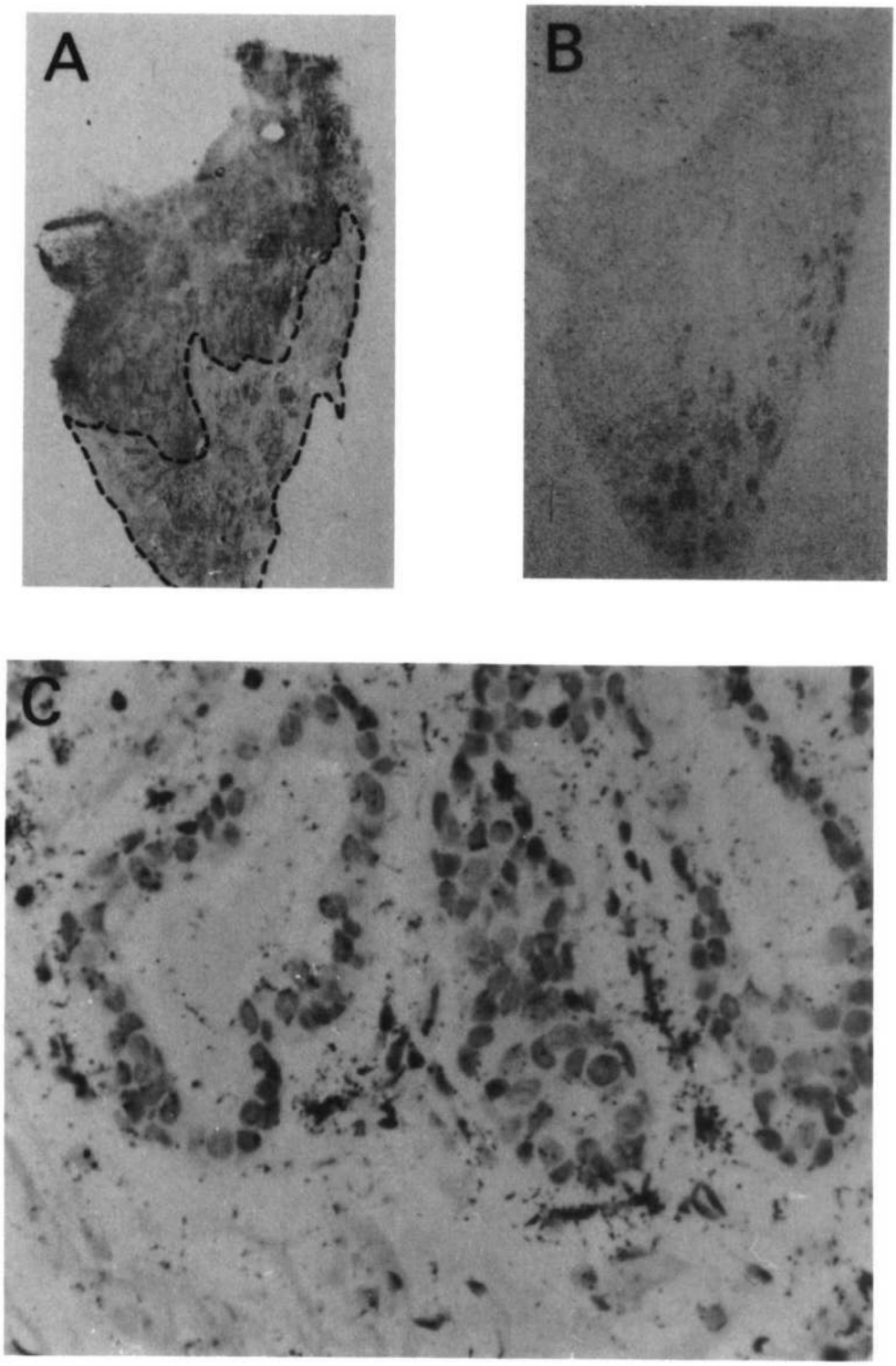

Fig. 4. *In Situ* Hybridization of Breast Cancer Tissues with IGF-I Probe

A, Histological section of breast cancer biopsy specimen; adjacent nonmalignant lobules are indicated by the *dashed line*. B, Autoradiogram of breast cancer tissues showed that the probe was localized to the areas of nonmalignant lobules. No specific hybridization was seen when using a labeled sense probe. The autoradiogram was a 2-week exposure of the slide on Hypermax film (Amersham). C, The nonmalignant lobule demonstrated that IGF-I probe hybridizes to stromal cells.

can be concluded that IGF-I is an autocrine growth factor for malignant epithelial cells *in vivo*. Although it is possible that an interaction between malignant epithelial cells and stroma is required before IGF-I can be expressed by the tumor, we have not observed this phenomenon in the *in situ* hybridizations we have performed.

Epithelial breast cancer cell lines express type I IGF

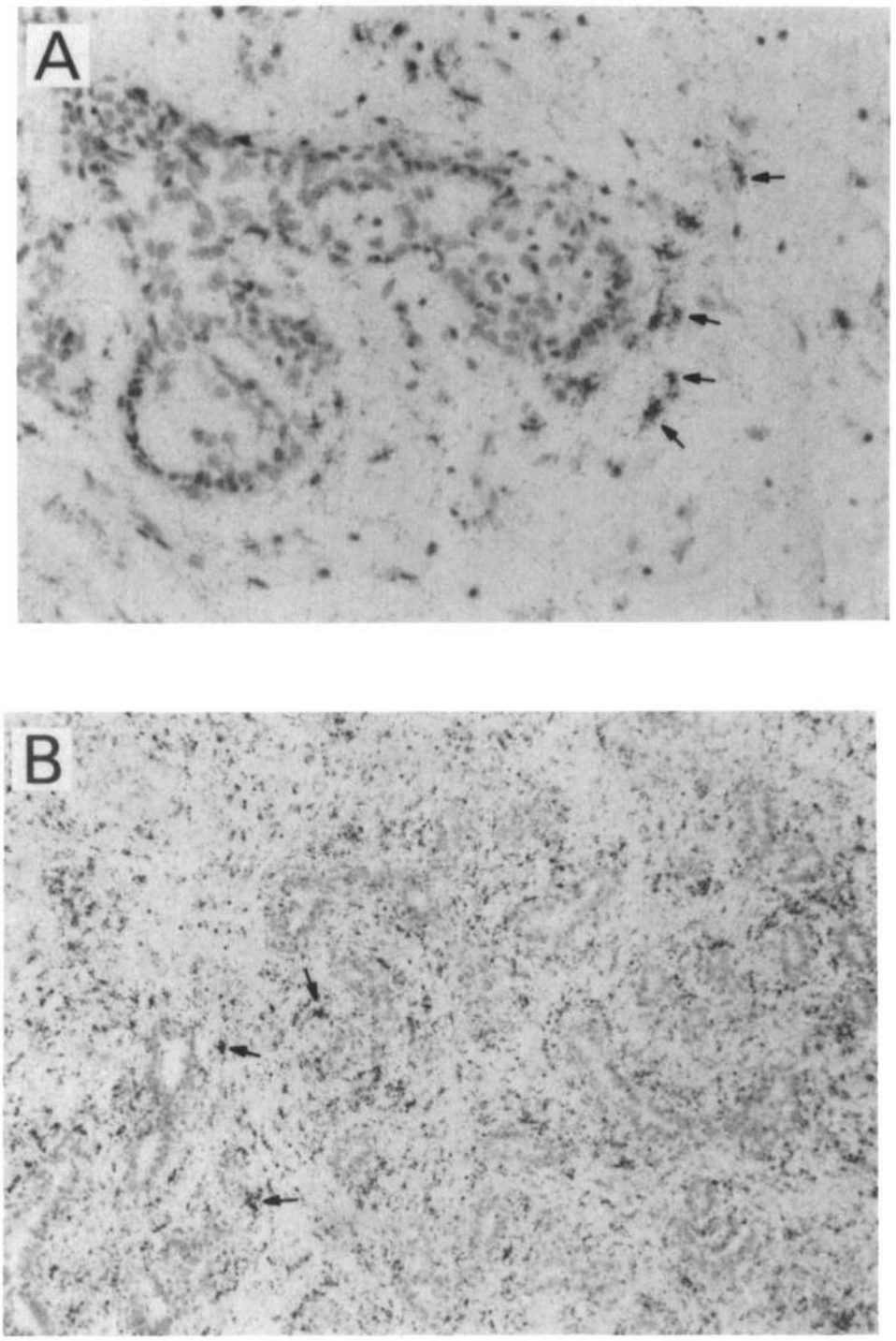

Fig. 5. *In Situ* Hybridization of Nonmalignant Tissues with IGF-I Probe

Arrows indicate silver grains exposed by the IGF-I probe. A, Histological section of a nonmalignant lobule taken from a breast cancer biopsy specimen. B, Fibroadenoma tissue also demonstrated localization of the IGF-I mRNA to stromal cells.

receptor RNA (Lebovic, G. S., unpublished data) and show specific binding for IGF-I (19). Since IGF-I is a potent mitogen for these cells (2), local stromal production of IGF-I could cause growth of breast cancer by paracrine stimulation. Of note is that IGF-I production by fibroblasts is enhanced by platelet-derived growth factor (PDGF) (9), and breast cancer cell lines have been shown to produce PDGF (22), yet have no PDGF receptor (Bronzert, D. A., personal communication). Therefore, in breast cancer, tumor production of PDGF could increase the production of fibroblast IGF-I to cause increased tumor growth. Authentic IGF-I may be a paracrine growth factor for breast cancer *in vivo*, and interactions between malignant epithelial cells and their surrounding stroma may play an important role in the growth regulation of breast cancer.

MATERIALS AND METHODS

Cell Culture

MCF-7 cells were originally obtained from the Michigan Cancer Foundation (Detroit, MI) (23). SK-N-MC, SK-N-SH, SY5Y, and BE2 were obtained from J. Biedler, Memorial Soan-Kettering Institute (New York, NY). C. Pat Reynolds (UCLA School of Medicine (Los Angeles, CA) provided the cell lines SAN, KAN, KCNR, A4563, and CHP-100. 6647 and TC32 were provided by T. Triche, UCLA School of Medicine (Los Angeles, CA). All other cell lines were obtained from American Type Culture Collection (Rockville, MD).

The medium used was Improved Minimal Essential Medium (Biofluids, Rockville, MD) with 10% fetal calf serum (Gibco, Detroit, MI), and cells were maintained at 37 C in a humidified atmosphere of 5% CO_2-95% air, as previously described (24).

RNA Preparation

Total cellular RNA from cell lines or tissue was isolated using the guanadinium thiocyanate method (25); concentration was determined spectrophotometrically and confirmed by agarose gel electrophoresis. Surgical specimens were frozen on dry ice immediately after dissection of the tissue. Tissue was stored at −70 C until RNA was prepared. All normal tissues were obtained as surgical biopsies. Normal breast tissues were obtained by dissection of grossly normal tissue away from breast tumor specimens.

IGF-I Probe

The IGF-IA cDNA was kindly provided by Ken Gabbay, Baylor College of Medicine (Houston, TX). The 540-bp *Pst*I-*Bam*HI fragment of IGF-IA cDNA was subcloned into pGem4 (Promega) after removal of 21 bp of the 5′ poly(G) tail. Labeled antisense RNA was transcribed using T7 polymerase according to instructions of the manufacturer. Unlabeled sense RNA was transcribed with SP6 polymerase, and the concentration was determined by spectrophotometer. [^{35}S]UTP (NEN, Wilmington, DE) was triple concentrated by lyophillization before synthesis of the probe.

RNAse Protection Assay

Hybridization and digestion conditions were performed as previously described (26). Twenty to 30 μg total RNA were hybridized with 5×10^4 cpm probe in 30 μl of an 80% formamide (vol/vol) buffer. Samples were hybridized for 12–16 h at 40 C, followed by digestion with RNAse A (Sigma, St. Louis, MO). The samples were extracted once with phenol-chloroform-isoamyl alcohol (20:20:1) and then precipitated with lug tRNA and 2 vol absolute ethanol. The pellets were lyophillized, then resuspended in an 80% formamide loading buffer. Protected fragments were visualized on a 6% polyacrylamide gel containing 8 M urea. The dried gels were exposed to x-ray film in the presence of a Quanta III (DuPont, Wilmington, DE) intensifying screen.

The sensitivity of the RNAse protection assay was determined by mixing serial dilutions of unlabeled sence IGF-I RNA with 30 μg tRNA. The conditions used to assay the standards were identical to those used to examine the cellular RNAs.

In Situ Hybridization

To preserve histological detail, only parafin-fixed tissue was used for *in situ* studies. Histological sections were deparafinized and rehydrated though progressive ethanol series. *In situ* hybridizations were performed as described previously (27). The sections were treated with 0.2 M HCl for 20 min, then denatured by incubation in 2 × SSC (300 mM NaCl, 30 mM Na Citrate) at 70 C for 30 min. Digestion with 0.5 μg/ml proteinase-K for 3 min was performed, followed by incubation for 30 min in 0.1 M Tris-0.1 M glycine. The sections were then washed in Dulbecco's PBS and acetylated for 10 min in fresh acetic anhydride diluted 1:400 in 0.1 M triethanolamine. The sections were dehydrated in an ascending ethanol series. Tissues were hybridized with 5×10^7 cpm/ml [^{35}S]UTP labeled antisense IGF-I RNA probes in 50% formamide, 10% dextran sulfate, 2 × SSC, 2 mg/ml BSA, 1 mg/ml salmon sperm DNA, 1 mg/ml yeast tRNA, and 50 mM dithiothreitol for 4 h at 50 C. Slides were washed with 50% formamide and 2 × SSC at 52 C for 25 min. Four washes of 2 × SSC at room temperature were followed by incubation in 20 μg/ml RNase A for 30 min at 37 C. The final wash was done in 50% formamide-2 × SSC at 60 C for 30 min. Sections were dehydrated through ascending ethanol series and then air dried. Sections were exposed to Hyperfilm-beta max (Amersham, Arlington Heights, IL) for 3 days before coating with NTB-2 emulsion (Eastman Kodak, Rochester, NY). If further washing was necessary, the sections were rehydrated and rewashed. After 3 weeks the emulsion was developed with D19 solution for 2.5 min, fixed for 3 min in Kodak fixer, and washed in water. Sections were stained with hematoxylin and eosin, dehydrated, and photographed. After examination, sections were then reexposed to Hyperfilm-beta max for 2–3 weeks.

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