RAPID COMMUNICATION

Rapid Detection of Xenotransplanted Human Tissues Using In Situ *Hybridization*

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A rapid and sensitive method for the identification of human tissues xenotransplanted in nude mice was developed. An *in situ* hybridization technique made it possible to distinguish between cells of human origin and cells of murine origin in formalin-fixed paraffin sections. High-molecular-weight DNAs extracted from human or mouse tissues were sonicated, nick-translated with ^{32}P-dCTP, and used as hybridization probes. Dot blot hybridization of ^{32}P-labeled probes revealed clear species-specific signals. Formalin-fixed paraffin-embedded tissue samples from repopulated tracheal transplants, containing either human tracheal epithelial cells or human renal tubular cells, were used. Cells of human and murine origin were distinguishable by *in situ* hybridization with sonicated DNA probes. This method has several advantages; simple preparation of probes, high sensitivity, and applicability to formalin-fixed paraffin-embedded tissue sections. *In situ* hybridization with sonicated DNA probes should provide a powerful tool for verifying the human origin of xenotransplanted tissues in nude mice. (Am J Pathol 1986, 122:386–391)

THE XENOTRANSPLANTATION of normal[1,2] and neoplastic[3,4] human tissues into nude mice has been successfully established as a useful method for evaluating the effect of carcinogens and/or tumor promoters.[5–7] Identification of tissue components of human origin in nude mice is sometimes difficult. Because there are no obvious histomorphologic differences between human and murine cells,[8,9] an *in situ* method is needed to aid in distinguising between cells and tissues of these two species. A method that would allow for the use of routine paraffin-embedded materials used in most pathology laboratories would be especially useful.

A few attempts for identifying the species of origin of cells in histologic sections of xenotransplants have been made, using fluorescent dye,[10] antisera against human fibroblasts,[11] and human keratin,[12] as well as antibodies to blood group antigens and to human T6 antigens.[13] These methods, however, have limitations and disadvantages, such as restricted tissue specificity,[11–13] some degree of cross-reactivity,[13] and the need for frozen sections[11] and fluorescence microscopy.[10,11]

An *in situ* hybridization technique is advantageous because it relates cellular structure to the molecular aspects of genes, providing a powerful tool for localizing specific genes on chromosomes, detecting viral genes in infected cells, and identifying specific cell types actively transcribing specific genes. Human and mouse genomes have been shown to contain a high degree of repetitive DNA sequences,[14–17] some of which are unique to these species.[15–17] These sequences contained in human genomic DNA could be used to demonstrate the human origin of tissues of questionable origin.

In this report, we describe a sensitive method for the detection of human tissues xenotransplanted into nude

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mice in paraffin sections by an *in situ* hybridization technique using sonicated genomic DNA probes.

Materials and Methods

Preparation of Human Tissues Xenotransplanted Into Nude Mice

Tissue samples were obtained from the xenotransplantation model developed by Klein-Szanto et al.[7] Human tracheal epithelial cells and human renal tubular cells from primary cultures[18] were inoculated into deepithelialized Fischer 344 rats tracheas transplanted subcutaneously into nude mice. These tracheas were fully repopulated 3–4 weeks after inoculation with either a normal human respiratory epithelium or a simple epithelium from human renal tubular cells. Some tracheal transplants with repopulated human tracheal epithelial cells were treated with 7,12-dimethyl-benz(a)-anthracene (DMBA) by insertion of a beeswax pellet containing 200 μg DMBA.[7] Squamous metaplasias were observed 4 to 8 weeks after carcinogen treatment.

For *in situ* hybridization, tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections (6 μ thick), prepared from 10 blocks embedded 2–10 months prior to sectioning, were mounted onto microscopic glass slides. Slides were precoated with Denhardt's medium (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.45 M sodium chloride, 0.045 M sodium citrate) and acetylated.[19] The sections were stored at 4 C until use.

DNA Extraction

High-molecular-weight DNAs were extracted from normal lungs of human fetuses and normal livers of SENCAR mice as described by Maniatis et al.[20]

Preparation of Hybridization Probes

Extracted human and mouse genomic DNAs were sonicated to less than 2.0 kilobase pair length, determined by agarose gel electrophoresis, and nick-translated with ^{32}P-dCTP (3200 Ci/mmol, ICN Radiochemicals, Irvine, Calif) by the technique described by Rigby et al.[21] Unincorporated nucleotides were removed by gel filtration over Bio-gel P-100. For *in situ* hybridization, ^{32}P-labeled DNA probe was ethanol-precipitated after addition of sonicated herring sperm DNA carrier. The DNA was pelleted by centrifugation in an Ependorf microcentrifuge for 15 minutes, and the DNA pellet was resuspended in the hybridization buffer to achieve a final concentration of 2 μg/ml. The specific activities of ^{32}P-labeled probes were 0.8–3.5 $\times$ 10^8 cpm/μg of DNA.

DNA "Dot-Blot" Hybridization

DNA "dot-blot" hybridization[22] was carried out to test the species specificity of the probes. Human and mouse DNAs were denatured, serially diluted, and spotted onto nitrocellulose filters. The filters were baked, prehybridized, and hybridized to 1 $\times$ 10^6 cpm of ^{32}P-labeled probe per milliliter of hybridization buffer. The filters were washed and then exposed to Kodak XAR-5 film at room temperature for approximately 8 hours by use of an intensifying screen.

In Situ Hybridization

In situ hybridization was carried out according to the method of Haase et al.[19] The sections were deparaffinized in xylene, washed in alcohol, and then air-dried. The sections were treated with 0.2 N HCl for 20 minutes at room temperature, incubated in 2× standard saline citrate (SSC) (1×SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) for 30 minutes at 70 C, treated with 1 μg/ml proteinase-K (Sigma Chemical Co., St. Louis, Mo) in 20 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ for 15 minutes at 37 C, and then dehydrated in graded ethanols (70% twice, 95% once, 5 minutes each). Cellular RNAs were digested with 100 μg/ml ribonuclease A (Sigma) and 10 units/ml ribonuclease T_1 (Sigma) for 30 minutes at 37 C, and the slides were subsequently postfixed for 2 hours at room temperature in a 5% solution of freshly prepared paraformaldehyde. After being washed in 2×SSC and water, the slides were transferred to 95% formamide, 0.1×SSC for 15 minutes at 65 C and ice-cold 0.1×SSC for 2 minutes to denature DNA and were then dehydrated in ethanol. The hybridization mixture contained 50% formamide, 10% dextran sulfate, 2×SSC, 10 mM Tris-HCl (pH 7.5), 1 mM ethylene diaminetetraacetate (EDTA), 1× Denhardt's solution (0.02%

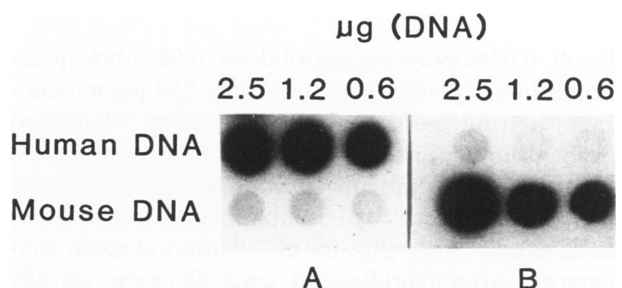

Figure 1—DNA dot-blot hybridization of human and mouse DNAs to ^{32}P-labeled sonicated human (**A**) and mouse (**B**) DNA probes.

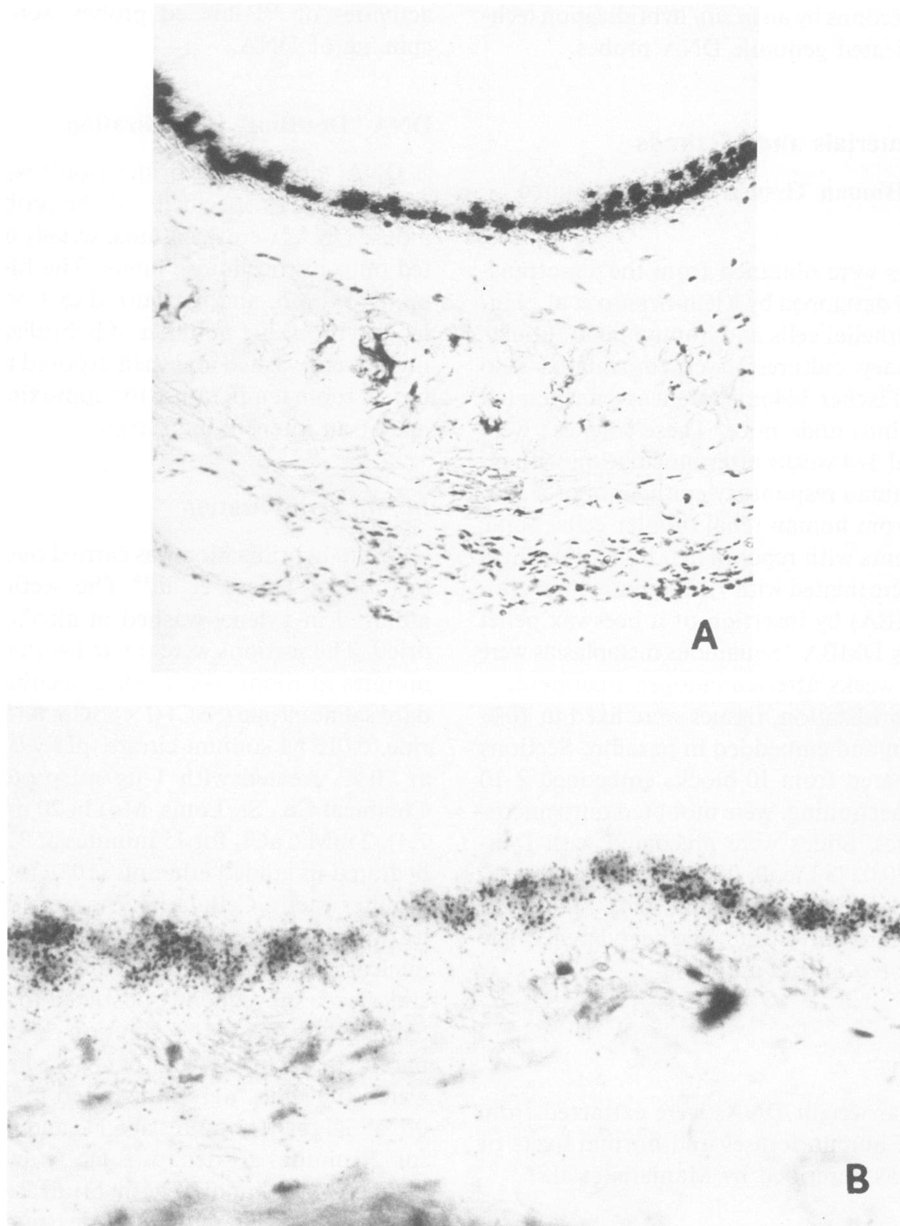

Figure 2—Demonstration by *in situ* hybridization with ^{32}P-labeled sonicated human DNA probes of human tracheal epithelial cels (**A**) (×100) and human renal tubular cells (**B**) lining the inner wall of tracheal transplants in nude mice (×340).

Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 100 μg/ml poly (A), 250 μg/ml sonicated herring sperm DNA, and 2 μg/ml ^{32}P-labeled probe. The mixture was denatured by heating for 30 seconds at 100 C and quickly cooled on ice. Each slide was incubated with 10 μl of mixture under 18×18-mm siliconized[20] converslips for 60–70 hours at room temperature. After hybridization, coverslips were carefully removed. The slides were washed in 50% formamide, 0.6 M sodium chloride, 10 mM phosphate buffer (pH 6.0), 1 mM EDTA for 1 hour at room temperature with two changes, dehydrated in graded ethanols containing 0.3 M ammonium acetate (70% ethanol, 5 minutes twice; 95% ethanol, 5 minutes once), and air-dried. The slides were coated with Kodak NTB-2 nuclear track emulsion, exposed for the appropriate amount of time at 4 C, developed with the use of a standard method, and counterstained with hematoxylin.

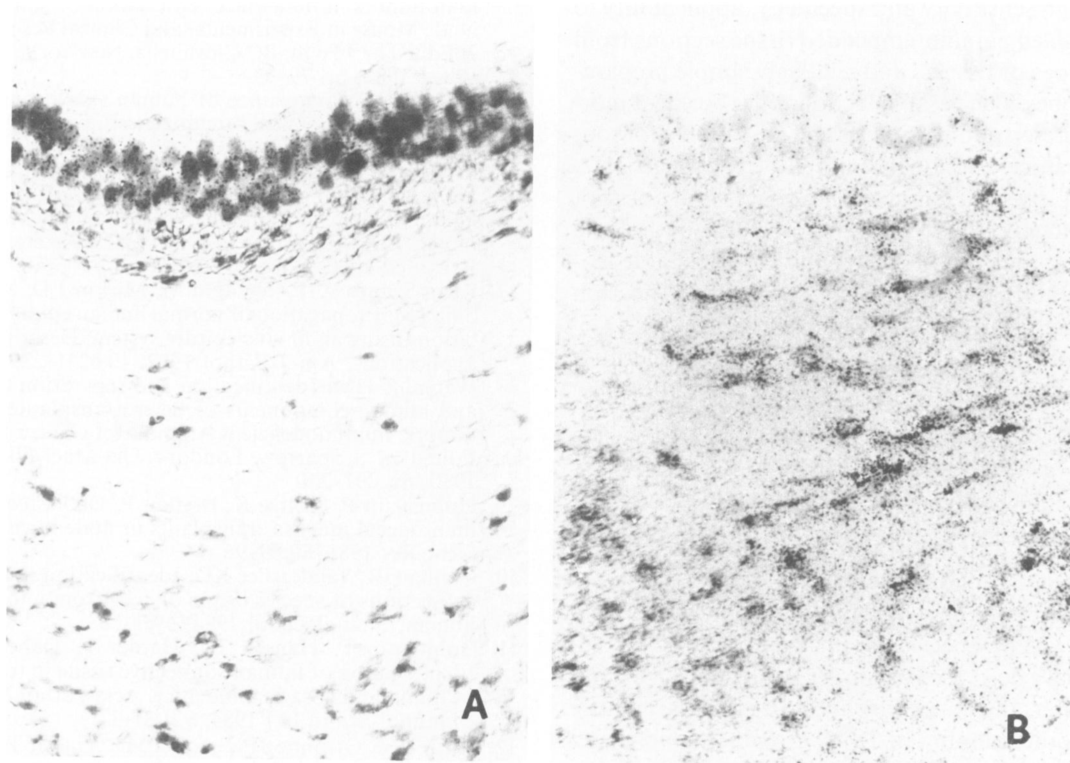

Figure 3—*In situ* hybridization of ^{32}P-labeled sonicated human (**A**) and mouse (**B**) DNA probes to the tracheal transplant with stratified metaplasia of reconstituted human tracheal epithelial cells treated with DMBA. (×280)

Results

DNA Dot-Blot Hybridization

Dot-blot hybridization was performed in order to determine the species specificity of hybridization of ^{32}P-labeled sonicated human and mouse genomic DNA probes to serial dilutions of both human and mouse DNAs. Autoradiography of dot-blot hybridization revealed high levels of hybridization of human and mouse probes to the homologous DNA, but very little hybridization to the DNA of other species (Figure 1). The significantly intense hybridization signals of sonicated DNA probes to the same species DNA indicated their usefulness as species-specific probes for human and mouse tissues.

In Situ Hybridization

In situ hybridization of sonicated DNA probes to paraffin sections of human cells xenotransplanted into nude mice was successfully achieved. Sonicated DNA probes enabled us to distinguish between cells of human and cells of murine origin. A high density of silver grains was observed over the human tracheal epithelial and renal tubular cells lining the inner wall of tracheal transplants when hybridized to the ^{32}P-labeled sonicated human DNA probe (Figure 2). Serial sections of the tracheal transplant, repopulated with human tracheal epithelial cells treated with DMBA, showed stratified metaplasias and demonstrated remarkable levels of accuracy in the labeling of cells according to probes and species of cells (Figure 3). Tissue sections gave consistently reproducible results. The reproducibility of results was independent of the age of the block, and the labeling intensity depended only on specific activities of the probes and the exposure time. A high-density accumulation of grains was achieved after short exposure time, ranging from 20 hours to 12 days. The sensitivity appeared satisfactory. The level of the background was generally low over the sections and almost undetectable outside the sections.

Discussion

The method described in this report for determining cells of human and murine origin by *in situ* hybridization with ^{32}P-labeled probes offers several advantages over the methods reported previously.[10-13,23] Advantages

include high sensitivity and specificity, applicability to formalin-fixed paraffin-embedded tissue sections from various types of tissues, and relatively simple preparation of probes. The paraffin sections offer excellent morphologic preservation of tissues and are suitable for routine laboratory use. Furthermore, the fact that DNA is a constitutive macromolecule of tissues that does not vary considerably with the physiologic or pathologic cell changes makes it an ideal constant and stable feature of cells on which to base any species identification assay.

Although ^{32}P-labeled probes produce a more diffuse localization of silver grains than ^{3}H-labeled probes, high specific activities of probes and efficient grain development can be achieved with ^{32}P-labeled probes, resulting in shorter exposure time.

The DNA of all eukaryotes has a high degree of repetitive organization[14-17]: approximately 20-30% of human DNA, for example, consists of repetitive sequences.[15] Many repetitive DNA sequences are species-specific.[15,17] The species-specific, repetitive DNA sequences have been used as probes to identify DNA and cells in filter and in *in situ* hybridization.[23-26] Those probes require cloning of DNA sequences and therefore are costly and time-consuming to prepare. We demonstrated by DNA dot-blot and *in situ* hybridization that sonicated human and mouse genomic DNAs could be used as species-specific probes. Further molecular characterization of the sonicated genomic DNA probes was not needed for the purpose of identifying human and mouse DNAs and was not carried out. Intense species-specific hybridization signals, produced by sonicated DNA probes, appear to be based on the higher relative amounts of species-specific repetitive DNA sequences. Although we examined this method in two types of human cells, including tracheal epithelial cells and renal tubular cells, it should be applicable to all types of tissues and cells except red blood cells.

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