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Keratin Gene Expression in Non-Epithelial Tissues

Detection with Polymerase Chain Reaction

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Keratin filament are characteristically present in epithelial cells and tumors, but have also been detected in many normal and neoplastic non-epithelial cell types using immunohistochemical techniques. To investigate the validity of this seemingly aberrant protein expression, we applied the highly sensitive polymerase chain reaction (PCR) technique to study keratin gene expression in a variety of non-epithelial tissues. Total RNA was extracted from nine samples of leiomyosarcoma, four non-Hodgkin's lymphoma, seven normal bone marrows, normal lymph node, normal peripheral blood cells, freshly isolated and cultured endothelial cells, cultured skin fibroblasts, and the myeloid leukemia cell line HL-60. Amplification primers and probes for the three most primitive keratin types (8, 18, and 19) were synthesized using published gene sequences. RNA from the breast carcinoma cell line MCF-7, known to be rich in all three keratins, was used as positive control. Concurrently run actin primers were used to confirm RNA integrity. After an initial cycle with reverse transcriptase, PCR amplification was performed for 30 cycles. Southern blots of the PCR products showed variably intense bands corresponding to keratin 8 and 18 gene products in all samples, offering conclusive evidence of keratin gene expression in cells of both stromal and hematopoietic derivation. However, keratin 19 gene transcription was not nearly so ubiquitous, being detected in normal fibroblasts and endothelial cells, two of four non-Hodgkin's lymphoma and four of nine leiomyosarcoma, but not in normal lymph node, peripheral blood cells, HL-60 cells, or any of the seven normal bone marrows examined. Dilutional experiments showed PCR to be highly sensitive in the detection of keratin 19 gene expression, capable of registering one MCF-7 cell in 10^6 HL-60 cells. These studies show that variable levels of keratin 8 and 18 gene expression may be detected by PCR in a wide variety of non-epithelial tissues, supporting previous immunohistochemical and phylogenetic studies. However, keratin 19 gene expression appears to be more restricted and was not evident in any hematopoietic cells devoid of contaminating stromal elements. These findings suggest a role for PCR in the detection of epithelial micrometastasis in certain sites, particularly bone marrow. (Am J Pathol 1993, 142:1111–1118)

Intermediate filaments (IF) are the principal components of the cytoskeleton in mammalian cells. Five major classes of IF exist and are believed to be expressed in a cell type-specific manner: keratin in epithelial cells, desmin in muscle cells, vimentin in mesenchymal cells, glial fibrillary acidic protein in glial cells, and neurofilaments in neural cells.[1] Because malignant cells tend to retain the IF of their progenitor cell type, immunohistochemical methods for the detection of these proteins have been widely instituted to aid in the characterization of neoplastic tissues.[2] Antibodies directed against keratin are the IF marker most commonly used and have proven to be reliable indicators of epithelial derivation.[3] However, recent

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studies have reported keratin immunoreactivity in smooth muscle tumors[4–8] and a diverse array of non-epithelial tumors,[9–21] suggesting this IF may not be as cell type-specific as previously thought. To investigate the etiology of this seemingly aberrant protein expression, we examined a variety of normal and neoplastic tissues of non-epithelial origin for keratin gene expression using the highly sensitive polymerase chain reaction (PCR) technique. Specifically, mesenchymal and hematopoietic tissues were studied for expression of the three most primitive keratin genes to test the specificity of the association between these IF and cells of epithelial origin.

Materials and Methods

Total RNA was extracted from recently isolated viable cells or a small piece (approximately 0.5 g) of frozen tissue stored at −80 C using commercial RNAzol B (Cinna/Biotex Laboratories International, Inc., Friendswood, TX). Nine cases of leiomyosarcoma, four malignant non-Hodgkin's lymphomas, seven normal bone marrows, normal peripheral blood mononuclear cells, freshly isolated and cultured umbilical vein endothelial cells, cultured normal skin fibroblasts, a normal lymph node, and the myeloid leukemia cell line HL-60 were extracted. RNA from the breast carcinoma cell line MCF-7 served as control for all studies. Using previously published complementary DNA sequences,[22–25] oligonucleotide primers and probes were synthesized by the phosphoramidite method on an Applied Biosystems DNA synthesizer (Foster City, CA). To avoid spurious amplification of genomic DNA, each primer set was chosen to span an intron.

Keratin 8 Primers and Probe Sequence

Keratin 8 primers and probe sequence were as follows. Primer I: 5′-CTG GTG GAG GAC TTC AAG AAC-3′; Primer II: 5′-GAC CTC AGC AAT GAT GCT GTC-3′; Probe: 5′-CAA GAA GGA TGT GGA TGA AGC-3′.

Keratin 18 Primers and Probe Sequence

Keratin 18 primers and probe sequence were as follows. Primer I: 5′-AGC CAT TAC TTC AAG ATC ATC-3′; Primer II: 5′-CTC TGT CTC ATA CTT GAC TCT-3′; Probe: 5′-ATT GTC AAT CTG CAG AAC GAT-3′.

Keratin 19 Primers and Probe Sequence

Keratin 19 primers and probe sequence were as follows. Primer I: 5′-GCG GGA CAA GAT TCT TGG TG-3′; Primer II: 5′-CTT CAG GCC TTC GAT CTG CAT-3′; Probe: 5′-CTT CCG AAC CAA GTT TGA GAC-3′.

Human β-Actin Primers

Human β-Actin primers were as follows. Primer I: 5′-TCA TCA CCA TTG GCA ATG AG-3′; Primer II: 5′-CAC TGT GTT GGC GTA CAG GT-3′.

One hundred nanograms of total RNA from each tissue sample were amplified using 10 pmol of each primer set specific for β-actin and keratins 8, 18, and 19. An initial cycle with reverse transcriptase was performed at 37 C for 7 minutes. The PCR reaction was then performed for 30 cycles using PCR buffer from a Perkin Elmer Cetus DNA Amplification Kit under the following conditions. 1) Keratin 8: denature at 94 C for 1 minute, anneal at 55 C for 1 minute, extend at 72 C for 2 minutes (extension time prolonged to 10 minutes for the last cycle). 2) Keratin 18: the annealing temperature was 48 C. 3) Keratin 19: the annealing temperature was 57 C.

After completion of the amplification cycles, 10 μl of each PCR product was run in 1.5% agarose gel, transferred to nitrocellulose membrane, and hybridized overnight with ^{32}P-labeled oligonucleotide probes internal to the PCR primers. Post-hybridization washes were carried out in 6X SSC at room temperature for 2 hours. Autoradiograph exposure times varied from 15 minutes at room temperature to overnight at −70 C.

To test the sensitivity of the PCR technique, MCF-7 cells were serially diluted with HL-60 cells. RNA extracted from each dilution was amplified and probed for the presence of keratin 19 transcripts.

Results

The primers used for amplification of keratins 8, 18, and 19 were proven to be unique by comparing their sequences with all known primate genes on file at the GenBank at the National Institutes of Health. No sequences homologous with any of the primers were found, except for the keratin genes specifically being amplified.

RNA integrity was confirmed in all samples by the detection of a 154-bp actin product in ethidium bromide-stained gels. Southern blot analysis of the

Table 1. *Keratin Gene Expression in Non-Epithelial Tissues*

Tissue sample	Keratin 8	Keratin 18	Keratin 19
MCF-7 cells	+	+	+
Peripheral blood mononuclear cells	+	+	–
Endothelial cells	+	+	+
Fibroblasts	+	+	+
Normal lymph node	+	+	–
Normal bone marrow (7)	+ (7)	+ (7)	– (7)
HL-60 cells	+	+	–
Non-Hodgkin's lymphoma (4)	+ (4)	+ (4)	+ (2)
Leiomyosarcoma (9)	+ (9)	+ (9)	+ (4)

MCF-7 is a human breast carcinoma cell line known to be rich in all three primitive keratins. HL-60 is a human acute myeloid leukemia cell line. For tissue types in which more than one specimen was studied, the number of samples with (+) or without (–) detectable transcripts is given in parentheses following the appropriate symbol.

amplified keratin 8, 18, and 19 gene products revealed variable keratin gene expression in the different tissue types, as summarized in Table 1. Only hybridizing bands of the expected molecular size (277 bp for keratin 8, 135 bp for keratin 18, and 214 bp for keratin 19) were taken to be conclusive evidence of keratin gene expression. Occasional higher molecular weight bands were seen on the keratin 8 and the keratin 19 blots and were taken to be nonspecifically amplified PCR products. All normal tissues examined, including isolated endothelial cells, cultured fibroblasts, normal lymph node, peripheral blood mononuclear cells, and the myeloid leukemia cell line HL-60, contained detectable keratin 8 and 18 transcripts (Figure 1). In addition, keratin 8 and 18 gene products were present in aspirated cells from all seven normal bone marrows studied (Figure 2). The intensity of the keratin 8 and 18 bands, a rough indicator of gene transcript levels best judged relative to MCF-7 expression, varied greatly between samples. Furthermore, due to the many vagaries involved in the process of RNA extraction, amplification, and Southern blotting, any quantitative estimate of gene product levels in these samples is presumptive, at best. However, overall there appeared to be more keratin 18 RNA present in these non-neoplastic tissues than keratin 8 RNA. The degree of expression of the two keratin genes was rarely equal in individual cases, and no consistent relationship of keratins 8 and 18 levels was evident in the different types of normal tissue. Even within the one normal tissue type on which multiple samples were studied, namely bone marrow, there were no consistently proportionate levels of keratin 8 and keratin 18 RNA.

All nine leiomyosarcoma samples and all four non-Hodgkin's lymphomas studied also expressed both keratin 8 and 18 genes (Figures 3 and 4). In these malignant tissues there was no consistent preferential expression of one keratin type, though in general there appeared to be greater keratin 8 levels in the majority of samples. As with the normal tissues, there was also no consistent relationship between tumor type and the relative expression levels of the two keratin genes.

In contrast to expression of the keratins 8 and 18 genes, keratin 19 gene expression is not nearly so

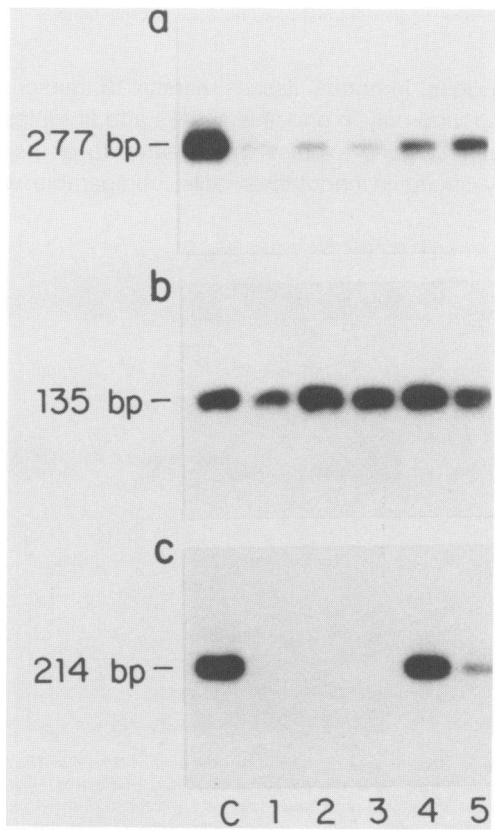

Figure 1. *Keratin gene expression in control MCF-7 cells* (**C**), *myeloid leukemia HL-60 cells* (**1**), *normal lymph node* (**2**), *peripheral blood mononuclear cells* (**3**), *endothelial cells* (**4**), *and fibroblasts* (**5**). **a**: *amplified keratin 8 gene product of 277 bp;* **b**: *135-bp keratin 18 product.* **c**: *214-bp keratin 19 PCR product. Note the presence of detectable keratin 19 gene product in the stromal cell samples but not in the hematopoietic samples.*

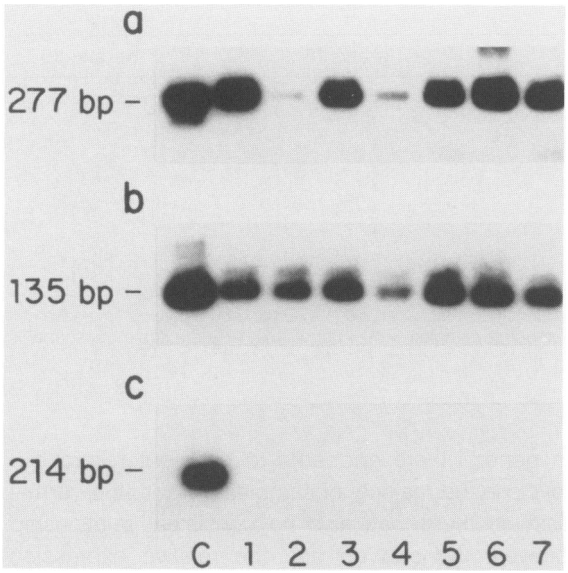

Figure 2. *Keratin gene expression in seven normal bone marrow samples.* a: *keratin 8 PCR product;* b: *keratin 18 product;* c: *keratin 19 product;* C: *control MCF-7 cells. Keratin 8 and 18 gene expression is relatively homogenous, except for two samples that show only small amounts of keratin 8 gene product. A spurious higher molecular weight band representing nonspecifically amplified product is present in sample 6 amplified for keratin 8. Note the absence of any detectable keratin 19 gene product in all the bone marrow samples.*

ubiquitous. In normal tissues, keratin 19 transcripts were found only in endothelial cells and fibroblasts. Keratin 19 gene expression appeared to be especially strong in endothelial cells, comparable with

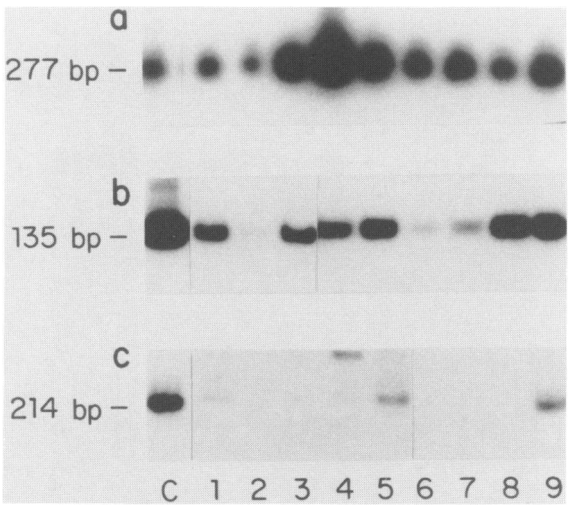

Figure 3. *Keratin gene expression in nine leiomyosarcoma. The panels and control tissue are the same as shown in Figures 1 and 2. There is variable but detectable expression of keratin 8 and 18 genes in all nine samples. There is a convincing keratin 19 signal in two cases (5 and 9), weak gene expression in two cases (1 and 4), and no detectable product in the remaining five specimens. Keratin 19-positive stromal cells (fibroblasts and endothelial cells) may be a source of contamination in these studies, particularly in cases 1 and 4. Rare nonspecifically amplified products were present after keratin 19 amplification, as seen in* lane 4; *these inappropriately sized bands were not considered to be indicators of gene expression.*

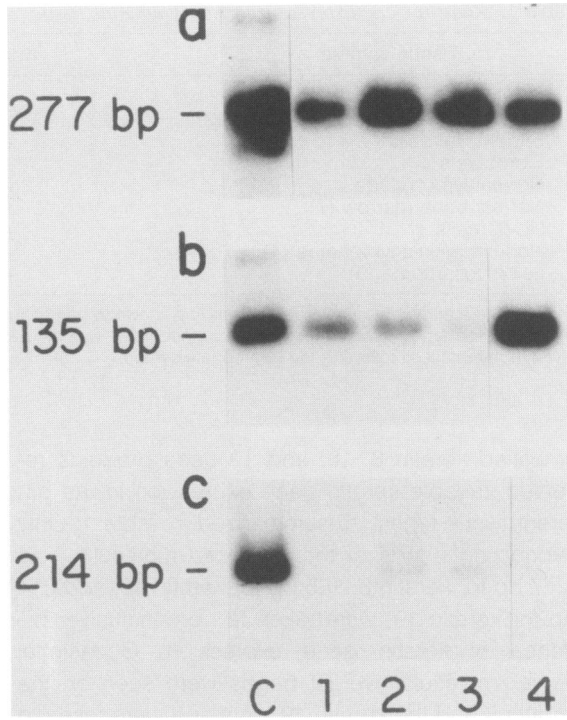

Figure 4. *Keratin gene expression in four non-Hodgkin's lymphoma. The panels and control tissue are the same as shown in Figures 1 and 2. There is variable expression of the keratin 8 and 18 genes in all samples. Keratin 19 gene expression is not detectable in two cases and shows only slight positivity in the other two samples. The faint signal seen in specimens 2 and 3 may be due to the presence of contaminating stromal cells.*

the levels found in MCF-7 breast carcinoma cells. Low-level gene activity was present in pure fibroblast cultures, but no keratin 19 transcripts were detected in HL-60 cells, normal lymph node, or peripheral blood and bone marrow cells (Figures 1 and 2). Keratin 19 gene expression was variable in the leiomyosarcoma samples, with two cases (5 and 9) being convincingly positive, two cases (1 and 4) showing faint bands, and the remainder being clearly negative (Figure 3). Keratin 19 transcripts were also inconsistent in the non-Hodgkin's lymphoma cases studied, with two cases (2 and 3) showing faint bands and two showing no bands (Figure 4).

The sensitivity of the PCR detection of keratin gene expressing cells was tested by diluting MCF-7 cells with HL-60 cells, and analyzing the extracted RNA for keratin 19 transcripts. As seen in Figure 5, the lower limit of detection was about one cell in 10,000 ($1:10^4$) after a 2-hour exposure at room temperature. However, overnight exposure at −70 C yielded faint bands at $1:10,^6$ a finding comparable with other PCR detection systems.

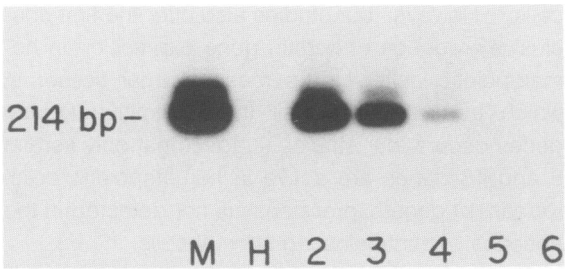

Figure 5. *PCR detection of keratin 19 gene expression in MCF-7 cells* **(M)**, *HL-60 cells* **(H)**, *and MCF-7:HL-60 dilutions of* $1:10^2$ **(2)**, $1:10^3$ **(3)**, $1:10^4$ **(4)**, $1:10^5$ **(5)**, *and* $1:10^6$ **(6)**. *Exposure of the autoradiograph for 2 hours at room temperature gives this picture showing detectable signals to the* 10^4 *dilution. However, overnight exposure at −70 C revealed a faint band in the* 10^5 *dilution.*

Discussion

The cytoskeleton of eukaryotic cells is composed of relatively large tubulin polymers (22–25 mm in diameter), microfilaments of actin (5–7 nm), and a third group of 7- to 11-nm diameter filaments called intermediate filaments, because of their size. Study of these intermediate sized filaments with human and rabbit antisera revealed significant immunophenotypic heterogeneity and allowed categorization into five classes with a high degree of cell-type specificity: cytokeratins in epithelial cells, neurofilaments in neurons and peripheral neuroendocrine cells, glial fibrillary acidic protein in astrocytes, desmin in muscle cells, and vimentin in mesenchymal cells.[1] With the exception of neurofilaments and cytokeratins, the intermediate filaments are composed of one type of protein. The keratin filaments are especially complex, however. At least 20 distinct cytokeratin proteins have been identified, each probably encoded by a separate gene.[26,27]

The keratin proteins may be divided into two broad subgroups based on their individual molecular weights and isoelectric points (pI), as determined by two-dimensional gel electrophoresis.[26] The smaller proteins, ranging from 40 to 56 kd and have relatively acid pI, comprise numbers 10 and 12 through 20 in Moll's classification system. The larger filaments range from 53 to 67 kd, have a relatively basic pI, and correspond to keratin types 1 to 8 according to Moll. A typical keratin filament is composed of one member from the smaller protein group and a specific corresponding member from the larger protein group. For example, keratins 8 and 18 are usually paired in simple normal epithelia, and are often abundant in epithelial neoplasms. Keratin 19 (40 kd) is the sole exception to this rule. This Moll type is the first to appear during embryonic life, and may nonspecifically pair with any one of a number of basic keratins.

Keratins are the most reliable markers of epithelial differentiation and are therefore more useful than the other intermediate filaments in the characterization of neoplastic tissues.[2,3] A large number of sensitive and specific antibodies to keratin filaments have been generated, leading to many reports on the utility of these reagents in diagnostic surgical pathology. Initial reports indicated a high degree of specificity of keratin antibodies for epithelial tissues.[26,28] However, as is common with immunoreagents, as the number of studies increased, the apparent specificity of the antibodies decreased. Keratin immunoreactivity has now been reported in a diverse array of normal and malignant non-epithelial tissues that include lymph node reticulum cells,[29] melanoma,[14] lymphoma and plasmacytoma,[12,16] and smooth muscle.[4,6,7] In addition, a long list of soft-tissue tumors have shown immunocytochemical positivity for keratin: synovial sarcoma,[10] epithelioid sarcoma,[9] leiomyosarcoma,[4,5,8] rhabdomyosarcoma,[15] malignant fibrous histiocytoma,[17] rhabdoid tumor,[11] Ewing's sarcoma,[13] endometrial stromal sarcoma,[21] and a variety of endothelial cell tumors.[18–20] Many of these tumor types have only rarely been reported to be keratin-positive, and the validity of some of these reports has been seriously questioned.[30–32] However, the aberrant expression of keratin filaments have been repeatedly documented in some tissues, such as normal smooth muscle, smooth muscle tumors, and endothelial lesions, with convincing biochemical confirmation in a few cases.[5–7]

To test the immunohistochemical evidence that keratin filaments are present in some non-epithelial cells, we examined a wide variety of normal and neoplastic non-epithelial tissues for keratin gene expression using the highly sensitive PCR technique. We restricted our study to keratins 8, 18, and 19, as these are the most simple keratins and are the first to be expressed in normal phylogeny.[1] These studies revealed that keratin 8 and 18 genes were transcribed in all tissues examined, both benign and malignant. Normal tissues positive for keratin 8 and 18 RNA included a variety of hematopoietic tissues in the form of peripheral blood mononuclear cells, aspirated bone marrow cells, and lymph node. Normal mesenchymal tissue examined included fibroblasts and umbilical vein-derived endothelial cells. Multiple cases of non-Hodgkin's lymphoma and leiomyosarcoma were the solid tissue tumors studied. Significantly, the HL-60 cell line, a pure culture of

acute myeloid leukemia, was also found to contain keratin 8 and 18 transcripts.

Although our results indicate that keratin genes 8 and 18 are ubiquitously expressed, keratin 19 gene activity appears to be somewhat more restricted. Specifically, no keratin 19 gene activity was detected in normal peripheral blood and bone marrow. Significantly, a total of seven normal bone marrow aspirations were studied, and no evidence of keratin 19 gene expression was detected, even after long exposure times at −70 C. Furthermore, the myeloid leukemia cell line HL-60 was devoid of detectable keratin 19 transcripts. Conversely, both of the normal mesenchymal tissue types studied, namely fibroblasts and endothelial cells, had detectable keratin 19 expression. A significant minority of leiomyosarcomas also expressed this keratin type, although the level of expression varied, as judged from the band intensity on the Southern blots. The presence of keratin 19 transcripts in two of the four non-Hodgkin's lymphomas is somewhat perplexing, especially considering the absence of detectable gene activity in peripheral blood and bone marrow, but may be explained on the basis of contaminating stromal cells (fibroblasts and endothelial cells). The faint positivity of the two positive cases is consistent with the presence of only a minor population of keratin-expressing cells in these tissues. Unfortunately, the ubiquitous presence of these stromal cells as a contaminating source of keratin 19 cannot be easily avoided. In fact, we cannot entirely exclude stromal cells as a possible source of keratin 19 transcripts in the leiomyosarcoma samples, even though the band intensity in two of the samples suggests that the source of this RNA is not a minor component of the cell population.

Detection of gene expression of simple keratins in a variety of non-epithelial tissues is a novel finding, but not entirely surprising, as good evidence of the filament in mesenchymal tissues already exist. The presence of keratin protein has been previously demonstrated in normal smooth muscle cells and smooth muscle tumors with Western blots.[5–7] In addition, although an early antibody study of umbilical vein endothelial cells showed no keratin positivity,[33] subsequent studies have found the protein in a subset of normal endothelial cells in the toad *Xenopus laevis*, in the rainbow trout, and in man.[34,35] These previous studies, as well as our detection of keratin gene expression in vascular endothelium, serve to illustrate the fact that endothelium is really a form of epithelium, being derived from mesenchyme that also gives rise to mesothelium and renal tubular cells.[36] However, our studies also offer the first conclusive evidence of keratin gene expression in hematopoietic cells. Unlike mesenchymal tissue, in which transcription of all three primitive keratin genes occurs, our studies suggest that only keratin 8 and 18 genes are active in hematopoietic cells. Keratin 19 gene expression was not detected in the absence of contaminating stromal cells.

As the function of intermediate filaments is still largely unknown, the biological significance of keratin gene expression in these non-epithelial tissues remains obscure. Their primary function has been presumed to be mechanical support, as these proteins are relatively insoluble and not easily degraded.[1] Maintenance of normal structure is important for all cells, and even though the protein may be present in only very small quantities, this seems the most likely role of keratin filaments in non-epithelial tissues. In epithelial cells, keratin filaments have also been shown to interact with desmosomal plaque proteins and are therefore probably important in cell-to-cell cohesion.[1] More recently, expression of primitive keratins has been correlated with invasive and metastatic potential in a series of melanoma cell lines, a cryptic but intriguing finding.[37]

This study was begun with the intent of evaluating the possible usefulness of PCR for the detection of epithelial micrometastases. Our results clearly indicate that the widespread presence of keratin 8 and 18 gene activity, even in hematopoietic cells and tissues, precludes the use of epithelial cell detection by PCR for these particular genes. However, keratin 19 gene expression is more limited and may be of some clinical utility, especially for the detection of small metastatic deposits in bone marrow. Unfortunately, the presence of keratin 19 activity in stromal cells is problematic, as these elements are inevitably present in solid tissue samples such as lymph nodes. The bone marrow specimens studied did not contain detectable keratin 19 messenger RNA, indicating that the act of aspiration apparently renders contaminating stromal cells below the detection limit of this technique. PCR analysis of keratin 19 may therefore be of some use in the study of aspirated bone marrow for micrometastasis, either as a staging procedure or in preparation for autologous bone marrow transplantation. Our dilution experiments show the sensitivity for the detection of keratin 19 gene expressing cells is high, capable of detecting one epithelial cell among 1,000,000 hematopoietic cells. This degree of sensitivity is probably near the limit of reliable PCR detection and is

comparable with that seen in the study of *bcl-2* gene rearrangements.[38]

In conclusion, these studies indicate that keratin filament gene expression is not restricted to cells of epithelial origin and may be found by PCR in a wide variety of normal and neoplastic non-epithelial tissues. In particular, our results lend credence to the previously reported finding of keratin filaments in smooth muscle and endothelial cells and tumors, and document for the first time keratin gene expression in hematopoietic cells. The ubiquitous presence of keratin gene expression, especially keratins 8 and 18, precludes PCR analysis of these genes as a detector of micrometastases. However, PCR analysis of keratin 19 gene expression may prove to be useful in the detection of epithelial tumor deposits in selected sites, such as bone marrow.

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