Expression of Oncogenes in Human Leukemias

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ABSTRACT

Oncogenes of retroviruses are directly related to cancers in animals; however, finding relevant associations with human cancer has been difficult. Studies reported here were designed to assess the extent of protooncogene transcription in human leukemias as compared to normal cells. Low-stringency hybridization of viral oncogene DNAs to cell messenger RNAs was done to determine whether evolutionarily divergent oncogene sequences were increased in leukemic cells. Several oncogenes hybridized at low levels to normal messenger RNAs. Some oncogene transcripts in fresh leukemic cells of 28 patients were present at higher levels than in normal cells. The oncogenes abl, erb, myc, and ras were expressed in all normals tested and a majority of the leukemic cells. However, fes, fps, and src probes hybridized rarely to transcripts in normal cells although more frequently to messenger RNA from leukemic cells. No oncogenes were expressed in specific patterns by cell types. It is concluded that transformation of human hematopoietic cells involves more than one oncogene and may include sequences unrelated to retroviruses.

INTRODUCTION

The oncogenes of retroviruses have been shown to cause tumors in animals (37), but whether or not these relationships hold for humans remains controversial. Since the recent understanding that the transforming sequences (oncogenes) are really cellular genes acquired by these viruses, it has seemed probable that the oncogenes may have more general functions as evidenced by expression in embryonic or regenerating tissues (12, 21) and are related to growth factors (9). Furthermore, normal cell homologues (c-oncns) have been mapped to specific human chromosomes (27).

In human cancers, the expression of oncogenes as mRNA has been demonstrated in a variety of tumor cell lines and fresh cells. Eva et al. (11) showed increased transcription of the myc, sis, bas, and abl oncogenes in human tumor cell lines. In several instances, the malignant cells had transcripts of different sizes than did normal cells. Westin et al. (39) reported transcripts of abl-like sequences in cells from a patient with AML and several cell lines. Although there were some minor differences in band intensity on northern blots, the same sizes of mRNA were generally seen in cells of myeloid or lymphoid origin. Likewise, the myc gene was expressed in normal and malignant cells. No correlation could be made between the level or type of transcripts and variety of transformed cells. The c-myb gene analogous to transforming v-myb of avian myeloblastosis virus was shown to be expressed at comparable levels in several stages of myeloid cells as well as blasts from a patient with AML and immature T-cells (38).

Robson and Tereba (26) hybridized RNA from fresh human leukemic cells with several hematopoietic-associated oncogenes under stringent conditions. Sequences homologous to myc were expressed in normal and leukemic cells over a wide range of concentrations. The myb probe hybridized more intensely to RNA from myeloid cells. Other oncogene probes for erb, rel, Hras, and src showed little hybridization. In general, there was no indication that any sequences homologous to these probes were expressed in a selective fashion for a given type of disease.

I have used several of the oncogene probes listed above as well as others to determine if sequences distantly related to oncogenes are expressed in human leukemias. Hybridization of mRNAs from normal lymphocytes and several types of lymphoid and myeloid leukemias was done under low-stringency conditions. As compared to Robson's study, there was more universal expression of erb, src, and ras genes. All of the oncogene probes except fps hybridized to some normal cell mRNAs. In general, the leukemic specimens showed a higher intensity of hybridization than did the normal. There was no specific pattern of oncogene hybridization to a given cell type or cell surface marker specificity. One case of ALL with the 9:22 chromosomal translocation had increased expression of abl transcripts.

MATERIALS AND METHODS

Leukemic cells were obtained from the bone marrow or peripheral blood of children and adults undergoing treatment at the University of Minnesota Hospitals. In all leukemia cases, the specimens contained over 90% blasts. Control samples were peripheral blood leukocytes. The RBC are lysed with ammonium chloride, and leukocytes are washed with Earle's balanced salt solution, pelleted, and lysed by a low-strength buffer with protease K (50 μg/ml) in the presence of bontonite or vanadylribonucleotide complexes (Bethesda Research Laboratories) (3). After digestion at 37° for 2 hr, the nucleic acids are deproteinized twice with phenol:chloroform stabilized with hydroxyquinoline (5). The aqueous layer is reextracted with chloroform:isoamyl alcohol and made 0.1 M in NaCl, and nucleic acids were precipitated overnight at −20° with 2 volumes ethanol. The DNA is washed out and RNA is pelleted by centrifuging at 10,000 x g for 30 min at 4°. The pellet is dissolved in sterile water, and DNA is treated with pancreatic RNase (boiled to inactive DNase) and then retreated with protease K, phenol:chloroform, etc., and ethanol is precipitated as before. DNA samples are digested with HindIII and the digested DNA was run on agarose gels or electrophoresed in a micropipet or with the Hybridot (Bethesda Research Laboratories) apparatus by methods described (34). Electrophoresis
and transfer of RNAs and hybridizations for "northern" blots was done as described by Thomas (34).

Oncogene probes and the suppliers are listed in Table 1. The cloned probes were amplified in their particular vector (λ or pBR322), excised with a specific restriction endonuclease, and separated by electrophoresis through 0.8% agarose. The oncogene insert was then purified by electrophoresion, phenol extraction, ethanol precipitation, and chromatography over DE52 (32). Probes were labeled in vitro with 32P nick translation to specific activities of 1 to 10^6 cpm/μg (19). Hybridization at low stringency [30% deionized formamide, 1 M NaCl, 0.02 M phosphate (pH 6.8), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate, and depurinated calf thymus DNA (500 μg/ml)] is carried out at 37° for 20 to 24 hr as described by Howley et al. (15) in the presence of 10% dextran sulfate (36). High-stringency hybridization was done at 41° with 50% formamide, 1 M NaCl, and the other components listed for low stringency.

For the fes and fps probes, similar sequences as did normal human RNA. Yeast tRNA did not hybridize with the abl probe. For the abl probe, the fes and fps probes, similar results were obtained although the relative intensity of hybridization was lower. Minimal hybridization was found with calf thymus DNA and none to the calf thymus or yeast RNAs except fps which showed minimal hybridization to calf thymus RNA.

Expression of Protooncogenes in Patient Samples. Low levels of hybridization were found between various oncogenes and mRNA from normal lymphocytes (Fig. 4). One specimen hybridized more than others and may have been related to recruitment of a certain cell type during leukapheresis or because of an intercurrent viral infection. Normal cells express levels of abl comparable to that seen in leukemic cells with some variation from patient to patient. Fes, fps, and src hybridized very weakly or not at all to normal transcripts.

Fig. 5 shows a panel of mRNA dot blots from various pediatric and adult patients with ALL. Patients 1 and 3 had "null cell" ALL, were common ALL antigen positive, and had no specific chromosomal abnormalities. Their mRNA had prominent hybridization to erb, fps, myc, or src. Patient 2 was a 23-year-old woman with Philadelphia chromosome-positive (t9;22) ALL who had mRNA species which hybridized extensively with the abl and Ki-ras probes. This is of interest because of human c-abl being located on chromosome 9 (13). Patients 4 and 6 are ones with the t4;11 chromosomal abnormality, a group of ALL patients known to have a poor prognosis (1). No unique pattern of oncogene hybridization was evident when comparing these 2 with other

Table 1

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Virus</th>
<th>Host</th>
<th>Provided by</th>
</tr>
</thead>
<tbody>
<tr>
<td>abl</td>
<td>Abelson leukemia virus</td>
<td>Mouse</td>
<td>S. Aaronson (33)</td>
</tr>
<tr>
<td>erb</td>
<td>Avian erythroblastosis virus</td>
<td>Chicken</td>
<td>J. M. Bishop (35)</td>
</tr>
<tr>
<td>fps</td>
<td>Feline sarcoma virus</td>
<td>Cat</td>
<td>C. Skinner (38)</td>
</tr>
<tr>
<td>fes</td>
<td>Fugu fugu sarcoma virus</td>
<td>Chicken</td>
<td>M. Shibuya (31)</td>
</tr>
<tr>
<td>fps</td>
<td>Harvey sarcoma virus</td>
<td>Rat</td>
<td>E. Scolnick (10)</td>
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<tr>
<td>myc</td>
<td>MCH II</td>
<td>Chicken</td>
<td>M. Groudine (25)</td>
</tr>
<tr>
<td>src</td>
<td>Rous sarcoma virus</td>
<td>Chicken</td>
<td>T. Pansco (14)</td>
</tr>
</tbody>
</table>

Results

Because these experiments are done under low stringency, there was concern that the oncogene probes might cross-hybridize. Fig. 1 shows the results of hybridizations done using the abl, fps 3' and fps probes to 10, 50, 100, and 500 pg unlabeled DNA of myc, abl, fps 5', and fps 3', and baboon endogenous virus gag-pol genes. The fps 5' probe hybridizes extensively with fps DNA and less with the fps 3' sequence as have been reported previously (30). The fps 3' probe hybridized to itself and slightly to fps (Fig. 1). Under the low-stringency conditions, abl hybridized only to itself. Thus, no unexpected cross-hybridization was found under these conditions.

Limited homology has been found between the probe containing erb A and B genes and leukemic mRNAs under stringent conditions (26). To establish if more distantly related proto-erb A and B genes are being expressed, the hybridization of erb genes was done at conditions of high and low stringency. As seen in Fig. 2, the panel of mRNAs demonstrated little homology to erb A and B at high stringency. However, at low stringency, mRNAs of 4 controls, 8 ALL, 2 AML, and 2 CLL patient samples hybridized with the probe which carries erb A and B genes.

Likewise, only one ALL patient's mRNA hybridized with the c-myc probe at high stringency. Under relaxed conditions, 4 normal and most of the leukemic patients' samples hybridized to c-myc. Others have reported hybridization of oncogene probes to mRNAs of normal and malignant cells under high-stringency conditions (11, 26, 38, 39). It would appear from the results in Fig. 2 that there are protooncogene sequences in human leukemic cells which are distantly related to the oncogene probes.

To determine the relative amount of protooncogenes in various human mRNAs, dilutions of the unlabeled probe and nonhuman sources of RNA and DNA were hybridized with the abl, fps, and fps probes (Fig. 3). In the normal mRNA sample, hybridization is barely visible on the original autoradiogram but not in the photograph. However, several leukemia patients' mRNA had greater hybridization. An ALL patient had the same relative hybridization as 50 pg of abl probe. AML and AMML patients had nearly 5 to 10 times as much hybridization as the normal cell RNA. Calf thymus DNA and RNA had about the same amount of abl sequences as did normal human RNA. Yeast tRNA did not hybridize with the abl probe. For the fps and fps probes, similar results were obtained although the relative intensity of hybridization was lower. Minimal hybridization was found with calf thymus DNA and none to the calf thymus or yeast RNAs except fps which showed minimal hybridization to calf thymus RNA.

Fig. 4. Hybridization of various oncogene probes to mRNAs from normal lymphocytes. The samples were collected either from peripheral blood (20 to 50 ml) or from lymphocytes isolated by leukapheresis. mRNA was isolated and blotted as described ("Materials and Methods") and hybridized under low-stringency conditions with the 32P-labeled oncogene probes.
ALL patients. In each row, several of the other ALL patients' mRNAs showed weak hybridization, but it was not above that seen in the normals.

In general, there was more extensive hybridization of oncogene probes to mRNA from patients with AML than from those with ALL (Fig. 6). The greatest hybridization was to mRNA from an infant with a primitive myeloid leukemia (Patient 3) whose mRNA had sequences analogous to abl, fes, myc, and src. Patient 4 was a child with AML who had 150,000 WBC/dl but no unusual cell surface or chromosomal markers. Her leukemic blast mRNA showed the most prominent hybridization to abl, erbfesfpsmyc, and Ki-ras. The other patients with prominent hybridization to abl and src had AML with no distinguishing features.

Of all the patients surveyed, mRNA from the patient with AMML (Fig. 7) had the most extensive hybridization to most probes, especially abl, fes, fps, myc, and src. At the time this sample was obtained from this patient, she was undergoing induction but was refractory to treatment. Contrary to results reported by others (39), I did not find increased expression of abl in one CML patient. The fact that my patient was Philadelphia chromosome negative may be relevant since this rearrangement may be important for increased expression of abl (13). Of the 3 CLL patients surveyed, at least 2 showed some homology to several oncogenes, especially abl, myc, Ki-ras, and src. Burkitt's mRNA came from a lymphomatous involvement of the ovary and hybridized only weakly to fps and src but not to the myc probe.

Fig. 8 shows the results of northern blots for specimens of mRNA from patients with increased oncogene expression as found by dot blots. Four separate patients are represented. The Ki-ras probe hybridized to mRNA species between 5.5 and 3.8 kilobases in a sample from an ALL patient (Fig. 8, Lane 1). An AML patient's mRNA (Fig. 8, Lane 2) gave only the 3.8-kilobase band. Some degradation is evident in both of these blots; there are more divergent sequences than closely related sequences. The differences are most striking with the erb A and B probe. I found that under low-stringency hybridization conditions one or 2 normal cells and most leukemic cells expressed erb.

From these results as well as those reported by others (11, 26, 38, 39), it seems clear that a single protooncogene is not responsible for a given type of leukemia. Recent experimental evidence has supported the concept that more than one oncogene may be needed to transform a normal cell (18). Since the quantity of oncogene expression was not markedly different in leukemic cells over normals, in many cases these results suggest that other genes may be responsible for the malignant change. Ozanne et al. (23) found that in a cell line which expressed high levels of c-abl RNA the gene which transformed NIH-3T3 cells was not abl. Recently, a unique transforming gene has been identified in Burkitt's lymphoma (8).

There are well-established relationships between retroviruses and animal tumors. Two examples of many hematopoietic cancers are B-cell lymphomas of chickens (22) and plasmacytomas and animal tumors. Two examples of many hematopoietic can-

**DISCUSSION**

There are well-established relationships between retroviruses and animal tumors. Two examples of many hematopoietic cancers are B-cell lymphomas of chickens (22) and plasmacytomas of mice (17). Except for the human T-cell leukemia virus, no complete retrovirus has been isolated from humans (24). Since the retroviral oncogenes are cell derived, they have also been assumed that they would be important parts if initiating or maintaining neoplasia (37). Although there is no direct proof for this in human cancers, some intriguing possibilities have been found.

The transforming sequences of the EJ bladder carcinoma cell line are related to Ha-ras and contain a point mutation which changes the physical characteristics of its protein product (4). Sequences related to ras have been found in human lung and colon carcinomas (20). The promyelocytic cell line HL-60 contains several copies of the myc gene (6, 7). Recently, an amplified n-myc sequence was reported in neuroblastomas (28).

<table>
<thead>
<tr>
<th>Probe</th>
<th>Normal</th>
<th>ALL</th>
<th>AML</th>
</tr>
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<tbody>
<tr>
<td>abl</td>
<td>4/4</td>
<td>6/9</td>
<td>7/10</td>
</tr>
<tr>
<td>erbfesfpsmyc</td>
<td>1/4</td>
<td>2/5</td>
<td>2/5</td>
</tr>
<tr>
<td>myc</td>
<td>4/4</td>
<td>7/9</td>
<td>4/7</td>
</tr>
<tr>
<td>Ha-ras</td>
<td>3/4</td>
<td>2/5</td>
<td>2/5</td>
</tr>
<tr>
<td>Ki-ras</td>
<td>2/4</td>
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</tr>
<tr>
<td>src</td>
<td>0/4</td>
<td>4/11</td>
<td>5/11</td>
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\* 1x = 0.15 M NaCl:0.015 M sodium citrate.
REFERENCES


Fig. 1. Autoradiographs of blots comparing hybridization of oncogene probes at two stringency to one another. Various amounts (10 to 500 pg) of the oncogene insert DNA were isolated and spotted on nitrocellulose. The fps 5', fes 3', and abl probes were labeled with $^{32}$P and hybridized to the blots. BaEv, baboon endogenous virus.

Fig. 2. Autoradiographs of mRNA dot blots hybridized with erb and myc oncogenes under high and low stringency. Samples 1 to 4, mRNAs from normal lymphocytes; Samples 5 to 12, ALL; Samples 13 to 17, AML; Sample 18, CML; Sample 19, Burkitt's lymphoma; Samples 20 to 22, CLL; Sample 23, ALL; Sample 24, yeast tRNA. The same blot was used for high- and low-stringency of experiments in each case.
Fig. 3. Autoradiograph of abl, fes, and fps probes hybridized to patient samples and unlabeled probe to determine relative copy number of oncogene expression. NI, normal mRNA; CT DNA, calf thymus DNA; CT RNA, calf thymus RNA; Y RNA, yeast tRNA.
Fig. 5. Low-stringency hybridization of oncogene probes to mRNAs from leukemic cells of patients with ALL. All patients had common ALL except Patient 2 (Philadelphia chromosome-positive ALL) and Patients 4 and 6 (t4/11).

Fig. 6. Low-stringency hybridization of oncogene probes to mRNAs from patients with AML.
Fig. 7. Low-stringency hybridization of oncogene probes to mRNAs from patients with AMML, CML, CLL, and Burkitt's lymphoma (BL) (ovarian tumor).

Fig. 8. Size-fractionated blot hybridization of leukemic RNAs with K-ras (Lanes 1 and 2) and myc (Lanes 3 and 4) probes. RNAs in Lanes 1 and 3 are from the patients with ALL. RNAs in Lanes 2 and 4 are from 2 patients with AML. kb, kilobase.
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