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Possible Involvement of Rearranged Proto-oncogene in T Cell Malignancy

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Abstract: The retroviruses carrying *v-myc* and *v-raf* oncogenes were infected into fetal thymic organ culture (FTOC) to study the molecular mechanisms involved in T cell development. T cell lymphomas in the different stages of T cell development were obtained from this culture system. Interestingly, a few cell lines obtained from this system have a lack of transfected oncogenes, however these cells have the characteristics of transformed cells. In spite of the discrete phenotype of these transformed cell lines, the same pattern of recombination of endogenous *c-raf* genes was detected from Southern blot analysis. We suggest in this regard that the translocation event of thymocytes, or abnormal promoter activity, can cause lymphomagenesis by way of *c-raf*.

Key words: *c-raf*, fetal thymic organ culture, rearrangement, retrovirus, transformation.

Oncogenes are evolutionary conserved genes which have been identified because they induce cellular transformation, either when naturally incorporated into a retrovirus or when their DNA is transfected into tissue culture cells (Bishop, 1983). Retroviruses act as natural vectors for the transduction of at least some cellular genes, designated proto-oncogenes (Bishop, 1982; Varmus, 1982), which bring about malignant transformation of infected cells. Proto-oncogenes are a subset of cellular genes which are capable of inducing tumorous growth after incorporation into retroviruses, upon specific alteration in structure, (Der *et al.*, 1982; Reddy *et al.*, 1982; Tabin *et al.*, 1982; Taparowsky *et al.*, 1982) or regulation (Kelly *et al.*, 1983; Tabin *et al.*, 1982; Taub *et al.*, 1982) within the cell. The transforming ability of activated proto-oncogenes, the oncogenes (Bishop, 1983), generally appears to be dominant in cell culture assays, and distribution of tumor induction *in vivo* by the oncogene-carrying retrovirus follows single-hit kinetics. Several types of data suggested possible interaction of the *raf* and *myc* oncogenes in some tumors. A combination of both *raf* and *myc* oncogenes in an infectious murine retrovirus induced hematopoietic neoplasms in addition to less prominent fibrosarcomas and pancreatic acinar dysplasia 1 to 3 weeks after inoculation. The hematological neoplasms consisted of immunoblastic lymphomas of T- and B-lineage cells and erythroblastosis (Rapp *et al.*, 1985). Moreover, the avian homolog of *v-raf* and *v-mil*, is naturally linked to *v-myc* in the carcinoma virus MH1 (Coll *et al.*, 1984; Jansen *et al.*, 1984; Sutrave *et al.*, 1984), in which it presumably contributes to carcinoma induction, since avian acute leukemia viruses, which only contain *v-myc*, have a much lower incidence of this tumor type (Alexander *et al.*, 1979; Carr *et al.*, 1960).

A number of cell lines were established from *in vivo* or *in vitro* methods. Of them many lymphoma cell lines were achieved by using retroviral vectors. Retroviral vector has been used to insert *v-Ha-ras* and *v-myc* oncogenes into fetal thymic cell lines (Cattermole *et al.*, 1989). To understand the molecular events during thymic development, constructing a library of thymic cell lines arrested in each stage of development is of importance.

In the fetal mouse, progenitor cells from liver and bone marrow colonize the thymus from day 10 to 11 of gestation, and in a period of 5 days, expand and mature to form pools of competent helper and cytotoxic T cells (Kisielow *et al.*, 1984). The thymic microenvironment appears to play a major role in regulating the growth and development of early progenitor cells, but the mechanisms involved remain unknown. Organ culture of murine fetal thymus lobes at day 13 to 14 of gestation has been successfully used to follow the maturation of thymocyte precursors that have already seeded this tissue (Kisielow *et al.*, 1984). In this regard, the FTOC system is very useful for infecting the retrovirus carrying oncogenes, and to understand the molecular mechanisms involved in the development of thy-

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mocytes.

We have co-infected retrovirus carrying *v-raf* and *v-myc* oncogenes with helper virus *leuk* during FTOC, and obtained several transformed cell lines. During analysis of these cell lines, we found transformed cell lines lacking transfected oncogenes. In this report, we describe data from the analysis of these cell lines and possible involvement of a certain oncogene in T cell lymphomagenesis.

Materials and Methods

To establish the T cell lines arrested in the different stages of development, we used a FTOC method and a retroviral vector containing *v-raf* and *v-myc* oncogenes.

Fetal thymic organ culture (FTOC) (Mendel *et al.*, 1978)

Pregnant C57BL/6 mice were used for our FTOC system. Fetuses were removed from pregnant females at various days of gestation. Thymic lobes dissected from fetal mice at different days of gestation were placed on the surface of polycarbonate filters (Nuclepore, Pleasanton, CA, 0.8-μm pore size) that were supported on blocks of surgical gelfoam (Upjohn Co, Kalamazoo, USA) in 5 ml of medium in 6 well plates (Nunc, Rockidle, Denmark). The Iscove's modified Dulbecco's medium supplemented with L-glutamine and 25 nM HEPES (GIBCO, Gaithersberg, USA) was obtained commercially and the following products were added: penicillin (100 IU/ml), streptomycin (100 μg/ml), 12% heat inactivated (30 min, 56℃) FCS (GIBCO), and 0.03% glutamine. The cultures were grown in a humidified incubator in 5% CO_2 in air at 37℃. After 3 days, half of the culture medium was changed with fresh medium.

Viral infection in fetal thymus

The culture medium for NIH/3T3 cells producing J2 virus was Dulbecco's modified eagle medium (DMEM) supplemented with 10% calf serum. NIH/3T3 cells producing the J2 recombinant retrovirus (Rapp *et al.*, 1985) were grown in dishes, and the viral supernatant was removed from the culture medium after 24 h of culture under confluent conditions. One ml of viral supernatant was added to the fetal thymic organ culture. To enhance the efficiency of viral infection, we have co-infected the helpervirus, leuk (Rapp *et al.*, 1983). Fetal thymuses for infection were at 14 and 16 days of gestation. After 2 days infection, thymic lobes were suspended and plated on micro titer plates at a concentration of 10^5 cells/well. Half of the medium was changed with fresh medium every 5 days. After a month of culture, alived cells were analyzed, and maintained by continuous passaging. Cell lines 32.1-i and 32.1-d were obtained by the above method at 14 days of gestation, and the 16A5 cell line was also established from the 16th day of gestation. The J2 virus and *leuk* helper virus were kindly provided by Dr. Rapp of the National Cancer Institute (NCI), National Institutes of Heath (NIH), USA.

Southern blot analysis

Genomic DNA was isolated using a standard procedure (Maniatis *et al.*, 1989). DNA samples were digested to completion with the restriction enzymes described in the results. Normalized amounts of DNA digests were electrophoresed through 0.7% agarose gels, blotted onto nitrocellulose membrane (Schleicher & Schuell) and hybridized using 5×10^5 to 2×10^6 dpm /ml of denatured ^{32}P-labeled probe, at 42℃ overnight. Membranes were washed at 65℃ in 0.1×SSC, 1% SDS and exposed on Kodak XAR-5 X-ray film (Eastman Kodak, Rochester, USA).

Northern blot analysis

Total cellular RNA was isolated by a modification of described procedures (Chomczynski *et al.*, 1987). Briefly, cells were lysed in a buffer containing 4.5 M guanidium isothiocyanate, followed by homogenization, phenol/chloroform extraction, and ethanol precipitation at −20℃. Total RNAs (10 μg/lane) were electrophoresed on 1% agarosed-formaldehyde gel, blotted onto Genescreen Plus nylon membrane (New England Nuclear, Boston, USA) and hybridized as recommended for Genescreen Plus. The washing conditions were the same as described for Southern blotting and exposed as above.

DNA probes

The 1.5 kb fragment from *Hpa*I-*Eco*RI digestion with mouse *c-raf* cDNA, the 1.1 kb fragment from *Bam*HI-*Not*I digestion with MC-29 virus, 500 bp of the Sau3A1 fragment from murine T cell receptor (TCR) Cα genes, and the murine TCR Jβ1 fragment were used to detect endogenous *c-raf*, viral *v-myc*, and rearrangement patterns of TCR genomic genes, respectively. The probes for TCR were kindly provided by Dr. Ada, Kruisbeek of the NIH, USA. and *c-raf* and *v-myc* were kindly provided by Dr. Jacob Troppmair of the NIH, USA.

Oligonucleotides

Two oligonucleotide primers used in the PCR protocol were synthesized. For the 5′-primer which recognizes the antisense of a part of *gag* sequences in J2 virus, is 5′CTGACAGCTCTGATCGAGTCT3′. The 3′-

primer which recognizes the sense of a part of *raf* sequences in J2 virus, is 5'GACCCGATCCGAGTAGACAGC3'. The primers span the region of 93 nucleotides containing the *gag*, and 409 nucleotides containing the *raf* gene in J2 virus.

PCR assay

Genomic DNA (1 μg) from cells was placed into a 50 μl reaction mixture containing 1×PCR buffer, 1 μM of each primer, 200 μM of each dNTP (dATP, dCTP, dTTP, and dGTP) and 3 units of Tag polymerase (Perkin Elmer Cetus, Emeryville, CA). PCR amplification was carried out with a PCR machine (Perkin Elmer Co.) under the following amplification condition: 94℃; 7 min, and 94℃; 1 min, 55℃; 2 min, 72℃; 3 min for 30 cycles. PCR reactions were separated by electrophoresis on a 1.2% agarose gel in TAE buffer. Gels were stained with ethidium bromide and photographed. The 123 bp DNA ladder for size marker was commercially obtained from Gibco BRL.

Results

Different stages of rearrangement of T cell receptor genes in established cell lines

To procure T cell lines arrested in different stages of development, we made use of a FTOC system and J2 retrovirus carrying *v-myc* and *v-raf* oncogenes as described in the methods and materials.

Analysis of cell lines from different stages of T cell development, and rearranged patterns of the T cell receptor gene are known to be the most remarkable and accurate means so far. We analyzed some of the established cell lines, of them 32.1-i, 32.1-d and 16A5, as described in this paper, showed distinct phenotypes. From Southern blot analysis using the probe specific for the Jβ1 of the T cell receptor, the 32.1-i cell line showed germ line configuration the same size as that from liver DNA, while both 32.1-d and 16A5 cell lines have rearranged patterns as shown in Fig. 1A. Furthermore the rearranged patterns of 32.1-d and 16A5 cells were different each other. While the 32.1-d cell line has both alleles of rearranged band, the 16A5 cell line has only one band with one deleted allele.

From Northern blot analysis using the probe specific for the Cα of the T cell receptor gene, a 1.8 kb band the same as that of control cells was detected from both the 32.1-d and 16A5 cell lines, however the 32.1-i cell line has no transcribed band, as shown in Fig. 1B. From these data, it is obvious that the 32.1-d and 16A5 cell lines have a more differentiated phenotype than the 32.1-i cell line.

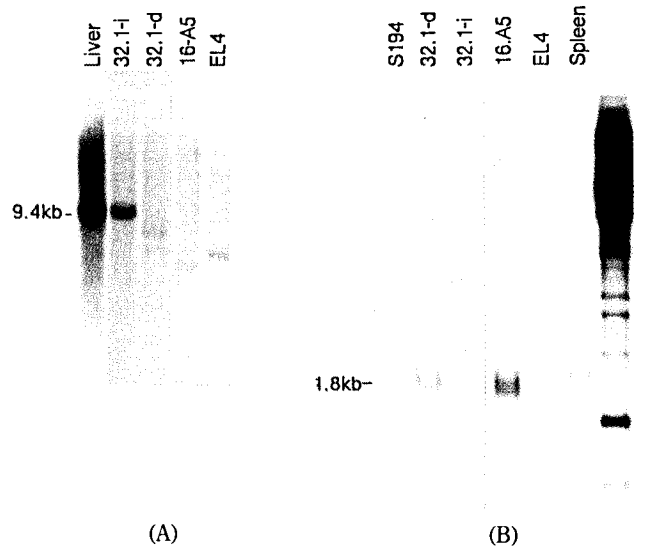

Fig. 1. Rearrangement and expression of T cell receptor genes. (A) Southern blot analysis of TCR gene in established cell lines. *Hin*dIII digested DNAs were electrophoresed, and hybridized with a ^{32}P-labeled Jβ1 probe. Liver and EL4 cells were used as negative and positive controls, respectively. 9.4 kb indicates the size of germ line configuration. (B) Northern blot containing 10 μg of total RNAs from each cell lines were hybridized with a ^{32}P-labeled Cα probe. EL4 and spleen cells were used as positive control, and S194 was for negative control.

A lack of transfected retroviral DNA in the established cell lines, 32.1-d and 16A5

To check integrated patterns of retroviral DNA, we have prepared DNAs from each cell line, digested them with restriction enzyme *Bam*HI, and hybridized them with a *v-myc* probe. From this analysis, only the 32.1-i cell line showed an integrated band, and not the other cell lines as shown in Fig. 2A. In addition, neither 32.1-d nor 16A5 showed the transcribed *v-myc* band in Northern blot analysis (Fig. 2B). To check whether or not only a part of transfected viral DNA has been deleted, we have carried out a polymerase chain reaction (PCR) test.

The designed primers span from the most 5′ of *gag* sequences to far down stream of *raf* sequences in J2 virus. As shown in Fig. 3, PCR products with these primers were detected only in 32.1-i cells.

Taking into account all of the above results, the 32.1-i cell line, which has an immature phenotype, alone contains the transfected J2 viral DNA. Other cell lines, such as 32.1-d and 16A5 lack transfected viral DNA in the genome. Furthermore, they have different T cell phenotypes from each other considering the stage of development at which they were arrested.

Specific rearranged pattern of endogenous *c-raf* DNA in the 32.1-d and 16A5 cells

DNAs from transformed cell lines were digested with restriction enzymes *Xba*I, *Eco*RI, or both *Eco*RI and

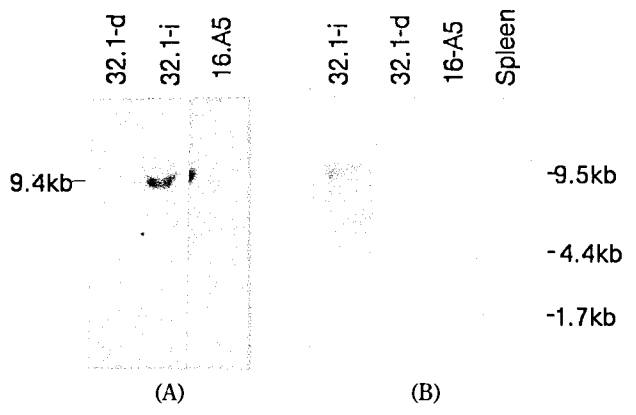

Fig. 2. The presence and expression analysis for the infected retroviral DNA. (A) Southern blot analysis of established cell lines. DNAs were digested with *Bam*HI and hybridized with ^{32}P-labeled probe specific for *v-myc*. (B) Northern blot analysis. 10 μg of total RNA from each of the cell lines were hybridized with ^{32}P-labeled *v-myc* probe. RNA from spleen cells was used as a negative control.

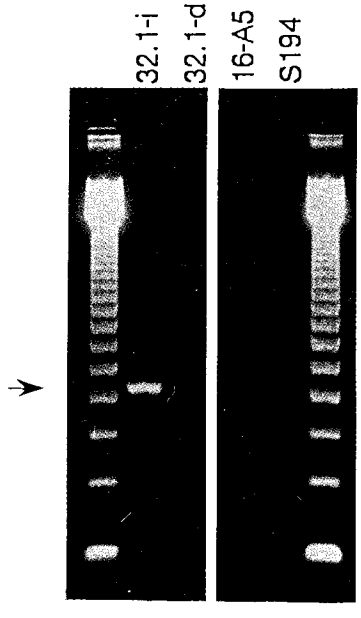

Fig. 3. PCR check for the transfected retrovial DNA. The primer sequences are described in materials and methods. The PCR product spans from 93 bp of the *gag* gene and 409 bp of the *raf* gene in J2 virus. The PCR product which was detected in 32.1-i is 502 bp in length. S194 cell was used as a negative control.

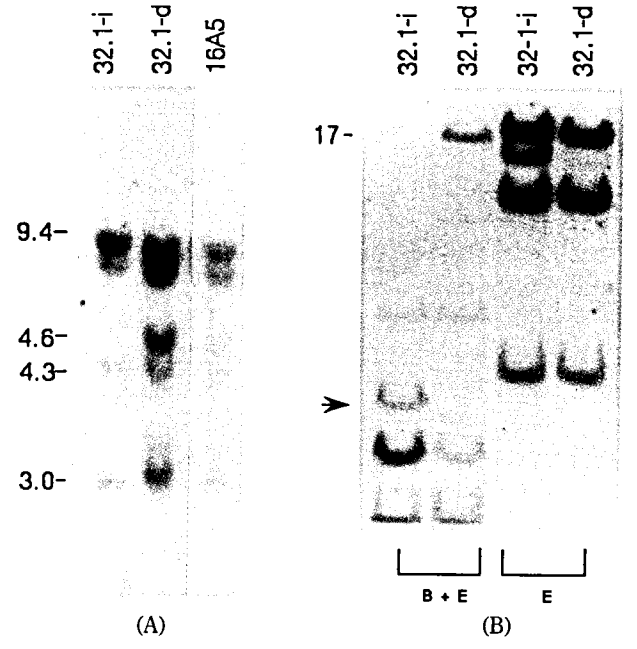

Fig. 4. Rearranged pattern of endogenous *c-raf* gene in both 32.1-d and 16A5 cells. DNAs were digested with (A) *Xba*I and (B) *Bam*HI plus *Eco*RI or *Eco*RI alone, and hybridized with ^{32}P-labeled probe specific for murine *c-raf*. In double digestion with *Bam*HI and *Eco*RI, the band indicated with the arrow in 32.1-i is rearranged to 17 kb in 32.1-d (B).

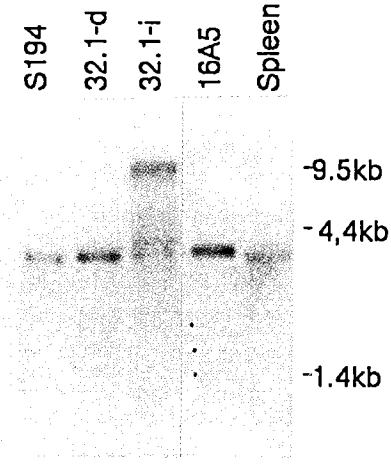

Fig. 5. Northern blot analysis of endogenous *c-raf* gene. 10 μg of total RNAs from each cell line were hybridized with ^{32}P-labeled murine *c-raf* probe. Control RNAs were from a S194 cell line and spleen cells.

*Bam*HI. Digested DNAs were then Southern transferred and hybridized with a murine *c-raf* probe. The 4.6 kb of rearranged band obtained from both the 32.1-d and 16A5 cells, and the rearranged pattern of these cells were identical to each other (Fig. 4A). The pattern of the endogenous *c-raf* band of 32.1-i obtained from Southern blot analysis was identical in size with the control DNA from cells such as S194 or EL4 cell lines (data not shown). It was of interest that the rearranged band from 32.1-d appeared when the DNAs were digested with *Eco*RI, or both *Eco*RI and *Bam*HI, as shown in Fig. 4B. From these results, we presume that the established cell lines, 32.1-d and 16A5, might be transformed by the rearrangement of a certain gene into the endogenous *c-raf* gene. The gene causing the transformation of T cells in this work remains to be identified.

No transcription of rearranged *c-raf* gene in the 32.1-d and 16A5 cells

We suspect that the oncogenesis of both the 32.1-d and 16A5 cells could be caused by the abnormal expression of a rearranged *c-raf* gene. To check this probability, Northern blot analysis was carried out, using the probe specific for murine *c-raf*.

As shown in Fig. 5, the transcribed bands from 32.1-d and 16A5 cells were shown with only one band, which is the same as the control RNA bands from S194 or spleen cells. The extra band, shown in 32.1-i cells, might be a cross hybridized from a transfected *v-raf* gene. The possible involvement of *c-raf* protein in the oncogenesis of 32.1-d and 16A5 cells could therefore be ruled out.

Discussion

We have used a mouse FTOC method and retrovirus to study the molecular mechanisms involved in each stage of T cell development. During analysis of established cell lines from this method, some transformed cell lines, which were lacking transfected oncogenes, were identified. We have focused on these two cell lines, 32.1-d and 16A5. These two cell lines were in different stages of T cell development. In spite of their distinguishing phenotypes, the two cell lines have the same rearranged pattern of endogenous *c-raf* gene. From the above results, we have a few questions. First, how could these cells be transformed without transfected retroviral oncogenes? Second, how is the rearranged *c-raf* involved in oncogenesis of thymocytes during viral infection of the FTOC system? The followings are possible events about which these questions can be proposed: First, a helper virus alone can induce oncogenesis. Previous studies have established that some of the helper murine leukemia viruses (MuLVs) are leukemogenic even when inoculated alone. In these leukemic tissues, their genomes have been found to activate endogenous proto-oncogene (Corcoran *et al.*, 1984; Gisselbrecht *et al.*, 1987; Li *et al.*, 1984; Selten *et al.*, 1984; Sheng *et al.*, 1984). However those papers reported the involvement of other oncogenes such as *myc*, *c-fms* and *c-myb* etc, concomitant with helper virus infection, but not the *c-raf* gene. Further, we couldn't find experimental evidence on the involvement of *leuk* helper virus in our transformed cell lines. From Northern blot analysis using *env* probe, which is specific for the murine leukemia virus, we could detect no significant band with the transformed cell lines 32.1-i and 16A5 when compared to control cell lines such as S 194, EL4 and spleen cell (data not shown). A second possibility is that the rearranged *c-raf* gene could be activated by some other strong promoter gene located on the proximal region of *c-raf*, and this abnormal expression of *c-raf* may play a role in oncogenesis. But this possibility also can be ruled out, because we couldn't find any distinguishable expression of *c-raf* RNA from Northern blot analysis in 32.1-d and 16A5 cells, compared to spleen cells as shown in Fig. 5.

Finally, we can suggest the possibility that a certain promoter adjacent to the rearranged *c-raf* induces abnormal expression of endogenous oncogene, and enables these cells to be transformed T cells. It remains to identify the genes involved in this lymphomagenesis.

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