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Research Article

Fibroblast growth factors (FGFs), such as basic FGF, have been implicated in the growth of Kaposi's sarcoma (KS) cells in vitro. In the evaluation of the expression of the various genes of the different members of the FGF family and their receptors in fresh KS tissue specimens, int-2 was found to be expressed in more than half of the KS tumors examined. Using reverse transcription PCR, the expression of int-2 was detected in 21 of 38 (55.2%) fresh KS biopsy specimens. In contrast, int-2 mRNA transcripts were not found in normal appearing skin from the same patients except in one sample which was obtained from an AIDS patient with disseminated KS lesions. Sequence data confirmed that the amplified sequences were derived from int-2 mRNA with proper splicing. In addition, 12 nucleic acid alterations were identified in eight out of nine KS tumor samples sequenced. Using immunohistochemical methods, int-2 protein was detected in some of the spindle-shaped tumor cells surrounding the abnormal endothelial-lined vascular slits histologically characteristic of KS. Int-2 specific immunostaining was shown to be present in both the nuclei and cytoplasm of these spindle cells but was more pronounced in the nuclei. Neither amplification nor gross rearrangement of the int-2 gene was detected in KS lesions by Southern blot analysis. These results suggest that the expression of int-2 may […]

Expression of Int-2 Oncogene in Kaposi's Sarcoma Lesions

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Abstract

Fibroblast growth factors (FGFs), such as basic FGF, have been implicated in the growth of Kaposi's sarcoma (KS) cells in vitro. In the evaluation of the expression of the various genes of the different members of the FGF family and their receptors in fresh KS tissue specimens, int-2 was found to be expressed in more than half of the KS tumors examined. Using reverse transcription PCR, the expression of int-2 was detected in 21 of 38 (55.2%) fresh KS biopsy specimens. In contrast, int-2 mRNA transcripts were not found in normal appearing skin from the same patients except in one sample which was obtained from an AIDS patient with disseminated KS lesions. Sequence data confirmed that the amplified sequences were derived from int-2 mRNA with proper splicing. In addition, 12 nucleic acid alterations were identified in eight out of nine KS tumor samples sequenced. Using immunohistochemical methods, int-2 protein was detected in some of the spindle-shaped tumor cells surrounding the abnormal endothelial-lined vascular slits histologically characteristic of KS. Int-2 specific immunostaining was shown to be present in both the nuclei and cytoplasm of these spindle cells but was more pronounced in the nuclei. Neither amplification nor gross rearrangement of the int-2 gene was detected in KS lesions by Southern blot analysis. These results suggest that the expression of int-2 may play a role in the pathogenesis KS by stimulating local angiogenesis and cell proliferation. (*J. Clin. Invest.* 1993. 91:1191–1197.) Key words: Kaposi's sarcoma • polymerase chain reaction • Int-2 • oncogene • fibroblast growth factors

Introduction

Kaposi's sarcoma (KS)[1] represents an opportunistic tumor of unknown etiology which is the most common neoplastic disorder seen in individuals infected with HIV, especially homosexual men (1). The characteristic histopathologic features of KS include bizarre shaped endothelial lined vascular slits surrounded by fasicles of spindle-shaped cells and a mononuclear cell infiltrate. In vitro studies on KS-derived cells have shown the potential roles of various cytokines and growth factors, including basic fibroblast growth factor (bFGF), IL-1β, IL-6, PDGF, and oncostatin M in the pathogenesis of KS (2–6). The FGFs are of particular interest since they are known to possess the ability to promote the proliferation of a variety of cells of mesodermal origin and have been shown to stimulate neovascularization, one of the primary characteristics of KS. Some of the members of the FGF family also induce cell transformation (7). Although bFGF has been implicated in the development of KS, its pathogenic role for KS in vivo remains to be determined since bFGF is also strongly expressed in normal skin and appears to be absent from the endothelial cells and the predominant spindle-shaped cells of KS lesions (8, 9). We have evaluated the expression of the various genes of the FGF family including aFGF, bFGF, int-2, HST/K-FGF, FGF5, FGF6 and their receptors, FGFR-1(flg) and FGFR-2(bek), in fresh KS biopsy specimens, uninvolved normal appearing skin from KS patients, or normal skin from healthy individuals. Although some of the other FGFs and the FGF receptors are expressed in some KS tissues (J. J. Li, et al., manuscript submitted), the most striking observation is that int-2 is expressed in more than half of the KS tumor tissues examined while almost no expression of int-2 was found in normal skin. To date there are no previous reports that show such a high prevalence of int-2 expression in adult human tissues.

Methods

Specimens. Samples of fresh KS lesions were obtained by either biopsy or autopsy. 10 normal appearing skin specimens were biopsied from HIV-1 positive individuals with KS and two biopsies of normal skin from HIV-1 sero-negative individuals undergoing plastic surgery (Table I).

Reverse transcription-PCR (RT-PCR). RNAs were extracted from either KS lesion or normal skin by acid guanidinum thiocyanate/phenol/chloroform extraction, and RT-PCR was performed as was described previously (10, 11). Briefly, 1 μg of total RNA was incubated with 4 U of ribonuclease-free deoxyribonuclease (Promega Biotec, Madison, WI) at 37°C for 45 min. The enzyme was subsequently heat inactivated (at 95°C for 5 min) and reverse transcription was carried out for 1 h at 37°C in a volume of 20 μl which contained 100 ng of random hexamer, 225 μM of each dNTP, 50 mM Tris-HCl (pH = 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 2 μg nuclease-free BSA, and 200 U of Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD). After boiling for 5 min, coamplifica-

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; KS, Kaposi's sarcoma; RT-PCR, reverse transcription PCR.

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Table I. Detection of INT-2 Expression in KS Lesions

Samples	Diagnosis	Number of specimens	Number of positive
Skin	AIDS-KS	27	12
	Classic KS	2	2
	KS (HIV-1 negative)	5	3
	Normal (HIV-1 positive)	10	1
	Normal (HIV-1 negative)	2	0
Lymph node	AIDS-KS	2	2
Tongue	AIDS-KS	1	1
Lung	AIDS-KS	1	1*

* The expression of int-2 was also detected from the skin of this patient.

tion was performed by adding 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 10 μg gelatin, 200 μM of each dNTP, 2.5 U of Taq DNA polymerase and primer pairs for both β-actin (5'-GAG,GAG,-CAC,CCC,GTG,CTG,CTG A-3' and 5'-CTA,GAA,GCA,TTT,GCG,-GTG,GAC,GAT,GGA,GGG,GCC-3') (12) and int-2 (5'-CTC,TAC,-TGC,GCC,ACG,AAG-3' and 5'-GGA,GGC,ATA,CGT,ATT,ATA-3'), (13) followed by 35 cycles of 94°C (1 min), 55°C (1 min), and 72°C (1 min). RT-PCR products were analyzed by 2% agarose gel electrophoresis, Southern blot hybridization with both [^{32}P]ATP end-labeled probes for β-actin (5'-GAA,ATC,GTG,CGT,GAC,ATT,AAG,-GAG,AAG-3') and int-2 (5'-GTG,GAT,CCG,CTC,CAC,AAA,CTC,-GCA,CTC-3'). After washing in 0.5 × sodium chloride sodium phosphate EDTA buffer (SSPE) (75 mM NaCl, 5 mM NaH_2PO_4, and 0.5 mM EDTA) at 55°C, autoradiograms were obtained by exposure of Kodak x-ray film at −70°C.

DNA sequencing. Bands of the predicted amplified int-2 cDNA fragment were purified and cloned into TA vector (Invitrogen, San Diego, CA). These fragments were then sequenced using ^{35}S-ATP and the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH). The reverse primer was also used to confirm the sequencing data.

Immunochemical staining. Sections (4 μm thick) from formalin-fixed KS biopsy specimens were cut, dehydrated in xylene, rehydrated followed by blocking the endogenous peroxidase with 1% hydrogen peroxide in methanol. The sections on slides were then sequentially incubated with normal rabbit serum at room temperature for 20 min, and a 1:1,000 diluted polyclonal sheep antibody to int-2 oncoprotein (Cambridge Research Biochemicals, Cambridge, MA) overnight at 4°C. An avidin-biotin-peroxidase technique (Vector Laboratories Inc., Burlingame, CA) was used employing rabbit anti-sheep reagents and 3,3-diaminobenzidine (14). The antibody to int-2, which was absorbed with human int-2 peptide, (Cambridge Research Biochemicals) was used as a negative control.

Southern blot hybridization. High molecular weight DNAs were prepared from KS lesions and normal skin by using the method previously described (15). DNAs were digested with PstI, electrophoresed on a 0.7% agarose gel and transferred to Gene Screen Plus membranes (Dupont, Boston, MA). The membrane was first prehybridized in buffer containing 1% SDS, 1 M sodium chloride, and 10% dextran sulfate at 65°C for 30 min and then hybridized in the same buffer with denatured [^{32}P]CTP-labeled BK4 probe (a 1.03-kb BamHI-Kpn fragment from human int-2, kindly provided by Dr. C. Dickson, Imperial Cancer Research Fund Laboratories, London) with salmon sperm DNA (100 μg/ml). Probe for fos gene was used as an internal control. After hybridization, the membrane was washed with 15 mM NaCl/1.5 mM sodium citrate, pH 7.0, containing 0.5% sodium dodecyl sulfate at 60°C for a total of 60 min with two changes of the washing solution.

Results

Int-2 expression in KS tissues was assessed by reverse transcription of the RNA followed by PCR (RT-PCR). The int-2 primer pair used for the PCR studies was chosen from different exons of int-2 to prevent amplification of any contaminating genomic DNA sequences. We used coamplification of RNAs of the int-2 gene and a reporter gene, β-actin, which enabled us to determine whether the RNA from the different samples studied were amplifiable.

RT-PCR products from KS tumors and normal skin specimens were analyzed by gel electrophoresis and Southern blot. Most of the samples revealed amplification of a β-actin fragment (834 bp) which demonstrated that the RT-PCR was successful. Amplified fragments of int-2 (252 bp) were found in more than half of the KS tissues examined. After Southern blot hybridization using int-2 and β-actin–specific probes and high stringency washes, appropriate bands were clearly detectable, which further confirmed that int-2 gene was expressed in these KS tumors (Fig. 1). Expression of int-2 was detected in 21 out of 38 of the fresh KS tumor samples (55.2%). In contrast, we were unable to detect int-2 mRNA transcripts in the normal appearing skin from the same patients except in one specimen which was obtained from an AIDS patient with disseminated KS lesions. Furthermore, we were unable to detect int-2 mRNA in normal skin from HIV-seronegative individuals. This result is consistent with previous observations that int-2 transcription is not detected in normal adult tissues (16).

To confirm further that the amplified products obtained were generated from int-2 mRNA, we purified the int-2 fragment from the gel, subcloned it into the TA vector (Invitrogen) and sequenced it. The sequence data obtained from nine different KS samples demonstrated that the amplified sequences were derived from int-2 mRNA with proper splicing. Surpris-

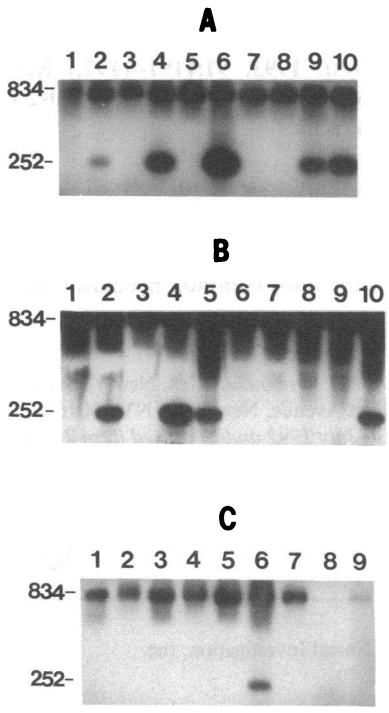

Figure 1. Representative autoradiograms of Southern blots performed on RT-PCR coamplification products hybridized with [^{32}P]ATP end-labeled probes for both β-actin and int-2. A 1-10, B1-5, and B10 are samples from KS lesions; B6, B9, and C1-9 are samples of normal appearing skin obtained from the same patients as B5, B10, and A1-9, respectively. B7 and B8 are samples from HIV-1–negative individuals. Since C8 did not show a β-actin band, the original sample probably did not contain intact RNA. The sizes of the specific fragments amplified are indicated.

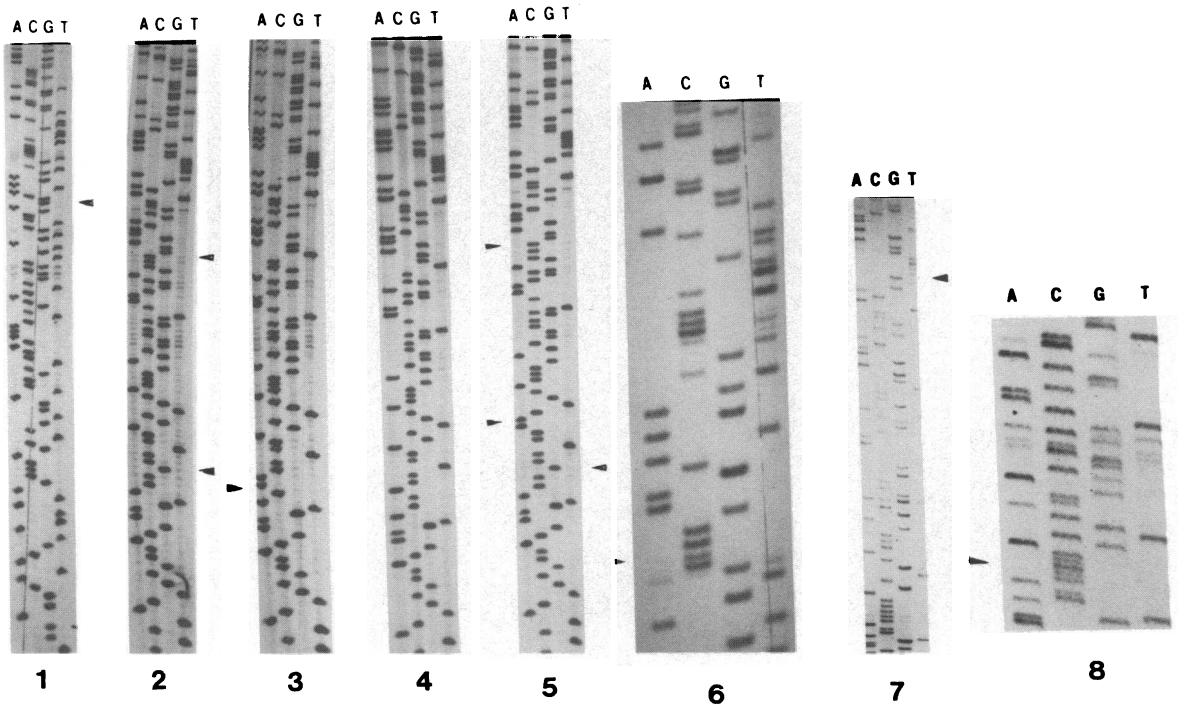

Figure 2. Sequence analysis of RT-PCR products of int-2 gene from eight KS lesions. Arrows show the altered base. The number of the sequencing gels are corresponding to that shown in Table II.

ingly, we found nucleic acid sequence alterations in eight out of nine KS tumor samples when compared with the published human int-2 cDNA sequence (13). The observed changes were confirmed by resequencing using a reverse primer. As shown in Fig. 2, the number of differences in an individual sample varied from 1 to 3, and a total of 12 alterations were identified in 8 of the 9 different KS specimens examined.

Most of the variations in int-2 cDNA sequences identified were located in exon 1, although four occurred in exons 2 and 3; three of which were near the splice sites. While two of the altered cDNA sequences would cause no change in the encoded protein, the others would result in amino acid substitutions. Some of these substitutions occur in a conserved domain of members of the FGF family (Fig. 3; Table II).

To determine whether the int-2 protein is also expressed in KS tissue, we performed immunostaining with antibodies against an int-2 peptide on formalin-fixed tissue sections from 10 KS biopsy samples. Three samples which showed expression of int-2 mRNA were also found to be positive. The int-2 oncoprotein was detected in some of the KS spindle-shaped cells, predominantly those found around the irregular-shaped vascular slits histologically characteristic of KS. Furthermore, int-2 specific staining was present in both the nuclei and cytoplasm with more pronounced expression in the nuclei (Fig. 4).

```
                 50        60        70   ↓    80        90        100  ↓  110       120
Wild Type:  LYCATKYHLQLHPSGRVNGSLENSAYS  ILEITAVEVGIVAIRGLFSGRYLAMNKRGRLYAS  EHYSAECEFVERIHELGYNTYAS
1.          ---------------------------  ------------------------------A-  ----------------------
2.          -------R----------P------   --------------------------------  ----------------------
3.          -------N-------------------  --------------------------------  ----------------------
4.          ---------------------------  -------K------------------------  ----------------------
5.          -------R-Q----------P------  --------------------------------  ----------------------
6.          ---------------------------  --------------------------------  G---------------------
7.          --------P------------------  V-------------------------------  ----------------------
8.          --------P------------------  --------------------------------  ----------------------

Wild Type:  part of published int-2 amino acid sequence(13)
1-8:        the alterations found in different KS lesions shown in fig.2
---         Indicate sequence data identical to wild type
↓           Represents the exon boundaries
            the amino acid sequence number of int-2 is shown above
```

Figure 3. Predicted substitutes of human int-2 amino acid sequence due to nucleic acid alterations.

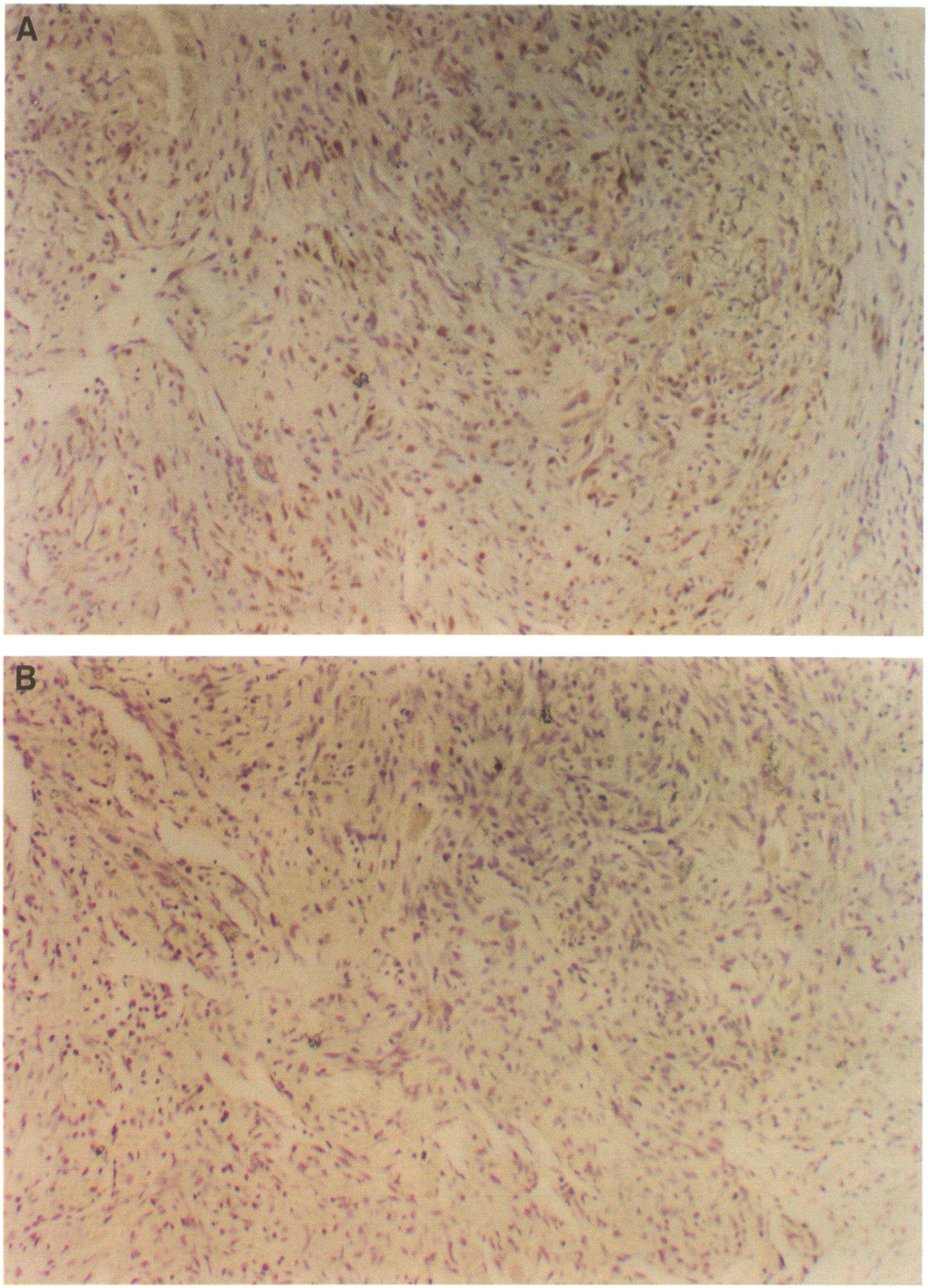

Figure 4. Immunostaining for int-2 protein in tissue specimens of KS lesions. (*A*) KS lesion with anti-int-2–positive brown stained spindle-shaped cells (×100). (*B*) Control immunostaining for int-2 protein in the presence of excess synthetic peptide (×100). (*C*) KS lesion showing positive perivascular spindle-shaped cell (×200). (*D*) High power view of anti-int-2 positive spindle shaped cells with nuclear and/or cytoplasmic staining (×400).

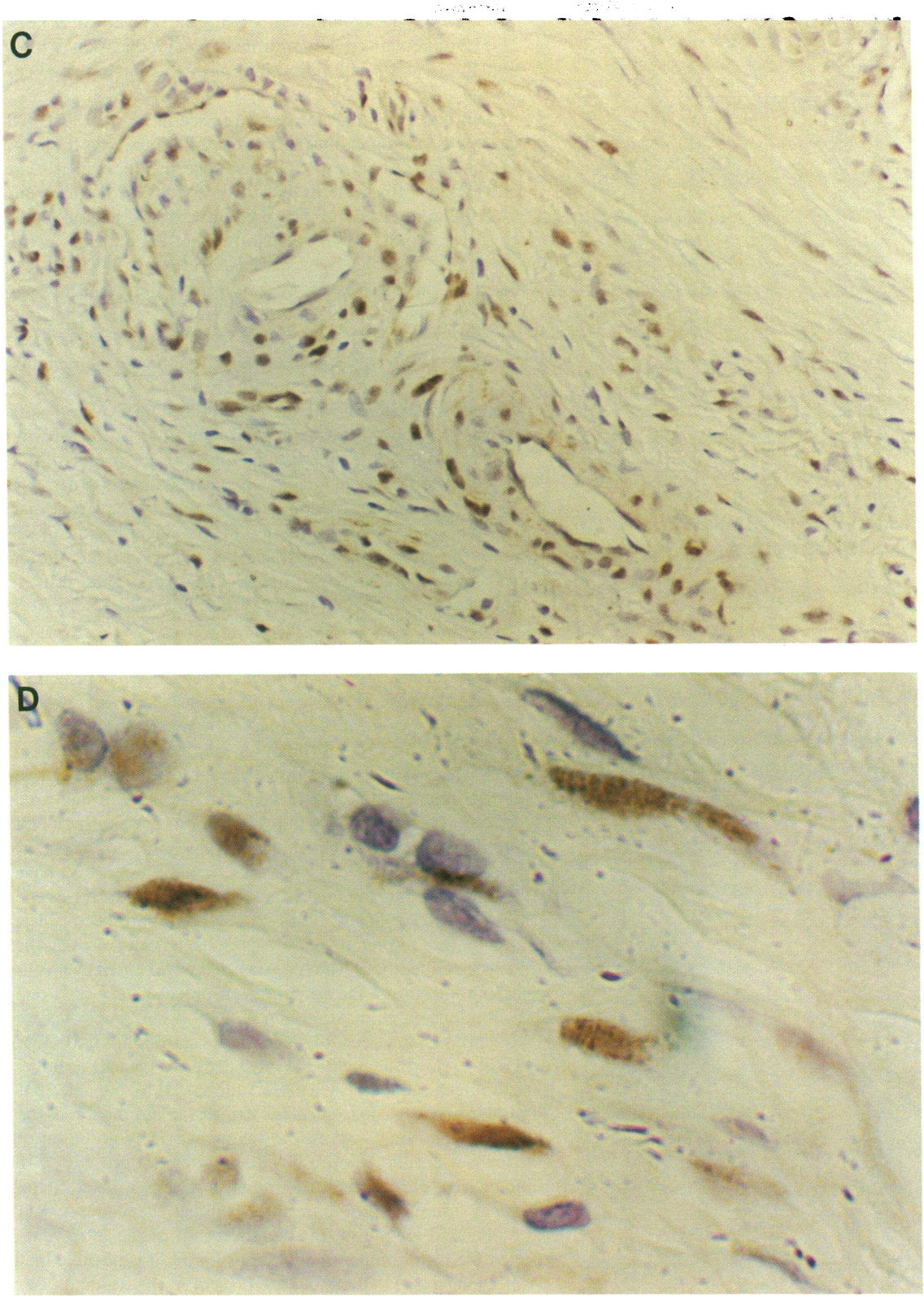

Figure 4 (Continued)

No positive staining for int-2 was observed in sections of normal skin.

The mechanism of activation of the int-2 gene is not clear. To test whether int-2 activation associated with gene amplification or rearrangement, genomic DNAs were extracted from KS and normal skin tissues, digested with restriction endonuclease PstI, Southern blotted, and hybridized with an int-2 specific probe. As shown in Fig. 5, each sample yielded a pattern of

Table II. Alterations of INT-2 Amino Acids in KS Lesions

KS lesion	Altered codon no.	Amino acid substitution
1	107	Ala → Ala
2	55	His → Arg
	68	Leu → Pro
3	55	His → Asn
4	82	Glu → Lys
5	55	His → Arg
	57	Gln → Gln
	68	Leu → Pro
6	109	Glu → Gly
7	56	Le → Pro
	75	Ile → Val
8	56	Leu → Pro
9	None	

bands of identical sizes when hybridized to the int-2 probe. The int-2–specific bands had equivalent intensities when normalized to a fos probe used as an internal control.

Discussion

Int-2 seems to be expressed primarily during embryonic development and has not been detected in any normal adult tissues (16–18). However, inappropriate expression of int-2 in the adult may have a role in the pathogenesis of certain tumors. The int-2 gene was originally identified as a site of frequent insertion of the murine mammary tumor virus which activated the transcription of the cellular gene leading to tumor formation in mice (19). Expression of int-2 in NIH3T3 cells has been shown to transform these cells (20). The oncogenic potential of int-2 has also been confirmed in transgenic mice (21). The amplification of int-2 gene has been described in 9–23% of human breast cancer specimens examined and the amplification of int-2 together with K-FGF has also been reported in a few other human carcinomas involving the head and neck (22–27). It still remains to be confirmed that amplification of int-2 actually coincides with transcriptional activation in these neoplasms.

Our data show that int-2 is expressed in > 50% of the KS specimens examined. Furthermore, int-2 protein was found to be expressed in both nuclei and cytoplasm. This is consistent with previous observations that an int-2 product resulting from AUG-initiation is found predominantly in the secretory pathway whereas the CUG-initiated form of int-2 was located in the nucleus in COS-1 cells transfected with int-2 cDNA (28). These findings suggest that the two forms of int-2 oncoprotein are also produced in KS lesions. The FGF receptors flg and bek are expressed in KS lesions. These receptors are able to recognize several members of the FGF family, although their affinity for the int-2 protein is still not yet clear. The coexpression of secretory int-2 protein with these receptors may provide a basis for autocrine and/or paracrine stimulation of KS cell growth in these tumors. In addition to its transforming activity, int-2 protein is related to factors having angiogenic properties. We observed that int-2 is predominantly expressed in perivascular cells of KS. The expression of int-2 thus could participate in KS tumor formation by stimulating local angiogenesis and tumor cell proliferation. Moreover the nuclear form of int-2 protein may play a role directly in the intracellular processes in KS oncogenesis.

The mechanism for the activation of the int-2 gene in KS tumor is not clear. It is possible that the activation of int-2 may be caused by specific viral agent(s), similar to the activation of int-2 in mice by murine mammary tumor virus. Epidemiological and laboratory data strongly suggest that KS may be associated with a sexually transmitted agent (29). Amplification or rearrangement of the int-2 gene could also lead to increased expression of int-2 mRNA in KS. However, our Southern blot data demonstrated that neither amplification nor gross rearrangement of the int-2 gene was present in KS lesions. We have identified a number of nucleic acid alterations in the int-2 gene in KS tissue. The fidelity of RT-PCR is an important consideration in the interpretation of these results. It is unlikely that the nucleotide alterations which we detected were caused by misincorporation during the amplification process, since only one silent point mutation was found in an FGFR-2 gene when we sequenced 18 different RT-PCR products from bFGF, FGF5, FGFR-1, FGFR-2, and P53 (exon 3 to exon 5) genes in parallel experiments (unpublished observations). It is possible that mutations of int-2 may effect the expression or function of the int-2 protein. Int-2 expression was increased by mutation of sequences around the presumed initiation codon in COS-1 monkey cells transfected with mouse int-2 DNA linked to the Simian virus early promotor (30). Although the sequence data we obtained are within the coding region of the int-2 gene, given the high number of alterations found, it is possible that other nucleic acid alterations are present in the regulatory sequences of the int-2 gene. The possible role of alterations of the int-2 gene in the pathogenesis of KS needs to be further evaluated.

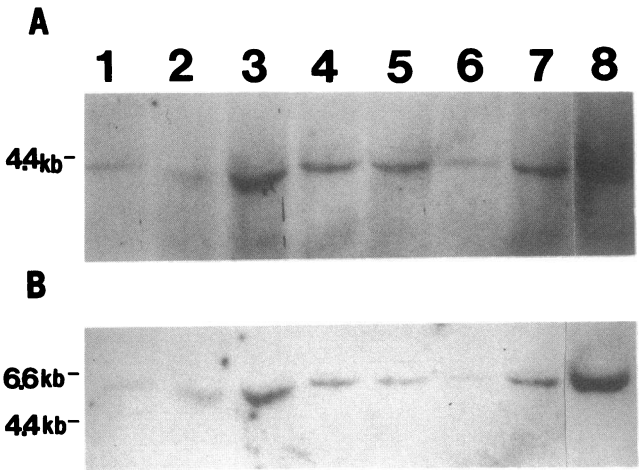

Figure 5. Southern blot hybridizations on KS lesion and normal skin DNAs. (Lanes *1–6*) DNAs from KS lesions. (Lanes *7* and *8*) DNAs from normal skin. (*A*) Southern blot probed with int-2 fragment. (*B*) Southern blot probed with fos fragment. DNA fragment sizes are shown on the left.

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