A CASP-8 Mutation Recognized by Cytolytic T Lymphocytes on a Human Head and Neck Carcinoma

By Susanna Mandruzzato,* Francis Brasseur,* Guy Andry,‡ Thierry Boon,* and Pierre van der Bruggen*

Summary

Of the antigens recognized on human tumors by autologous cytolytic T lymphocytes, all those defined thus far have been identified on melanoma or renal cell carcinoma. We report here the identification of an antigen recognized by autologous cytolytic T lymphocytes on a human squamous cell carcinoma of the oral cavity. The antigen is encoded by a mutated form of the CASP-8 gene. This gene, also named FLICE or MACH, codes for protease caspase-8, which is required for induction of apoptosis through the Fas receptor and tumor necrosis factor receptor-1. The mutation, which was found in the tumor cells but not in the normal cells of the patient, modifies the stop codon and adds an Alu repeat to the coding region, thereby lengthening the protein by 88 amino acids. The ability of the altered protein to trigger apoptosis appears to be reduced relative to the normal caspase-8. The antigenic peptide is a nonamer presented by HLA-B*3503. The five last amino acids are encoded by the extension of the reading frame caused by the mutation. This, together with previous observations of CDK4 and β-catenin mutations, suggests that a significant fraction of the point mutations generating a tumor antigen also play a role in the tumoral transformation or progression.

Several tumor antigens recognized by autologous cytolytic T lymphocytes (CTL) have been identified, both on mouse and on human tumors. The initial work carried out with mouse mastocytoma P815 revealed that antigens recognized on tumors by CTL can arise either from point mutations occurring in genes that are expressed ubiquitously or from the transcriptional activation of genes not expressed in normal tissues (1–3). Subsequent work provided two interesting examples of mouse tumor antigens resulting from point mutations (4, 5).

A number of human tumor antigens recognized by autologous CTL also belong to these two categories. Some of them are encoded by genes active in a significant proportion of tumors of various histological types but silent in normal tissues except in testis (6, 7). Others are encoded by mutated genes (8–11). In the case of melanomas, autologous CTL can also be generated against differentiation antigens present on both melanoma cells and normal melanocytes (12, 13). All these antigens were originally identified on melanoma and renal cell carcinoma; tumors that are considered sensitive to an immune attack.

Head and neck carcinoma has a worldwide incidence of ∼400,000 new cases per year. The most important risk factors are tobacco smoking and alcohol consumption. Head and neck squamous cell carcinoma (SCCHN)1 tumors are generally infiltrated by T lymphocytes (14, 15). Cytolytic T cell lines with a specificity for the autologous tumor were obtained from peripheral blood lymphocytes of a patient and from tumor-infiltrating lymphocytes in several other patients (16–18).

We have made a systematic effort to obtain tumor cell cultures from biopsy specimens of head and neck carcinomas. Permanent cell lines were established from 14 tumors, representing a 19% success rate. Using five of these cell lines, we performed mixed lymphocyte–tumor cell cultures by using irradiated tumor cells and autologous blood lymphocytes. With four patients, we failed to obtain T lymphocytes that lysed the autologous tumor cells. However, from the blood lymphocytes of patient BB49 we were able to isolate a CTL clone that lysed the autologous tumor cells. We report here the identification of the antigen recognized by this CTL clone.

1Abbreviations used in this paper: FADD, Fas-associated death domain protein; FasL, Fas ligand; HS, human serum; MLTC, mixed lymphocyte–tumor cell culture; SCCHN, head and neck squamous cell carcinoma; TRADD, TNFR1-associated death domain protein.
Materials and Methods

Cell Lines. Squamous cell carcinoma cell line BB49-SCHN was adapted to culture from a tumor relapse resected from the floor of the oral cavity of patient BB49, a 70-yr-old Caucasian woman in stage IV (T2N2M0), who died as a result of tumor progression 4 mo later. The tumor cell line was transfected by the calcium phosphate precipitation method with expression vector pEFBOS (received from S. N. agata, O. saka Bioscience Inst., O. saka, Japan), containing the coding sequence for human costimulatory molecule B7-1 (CD80) and in which the gene conferring puromycin resistance was inserted by J.-C. Renaud (Ludwig Inst., Brussels, Belgium; 19). Transfectants were selected with 0.5 μg/ml of puromycin and cloned by limiting dilution. Clonal subline BB49-SCHN.B7 was selected on the basis of a high expression of B7-1, as determined by cytofluorimetric analysis with mAb BB1 (Becton Dickinson, Mountain View, CA).

Culture media were supplemented with 10% FCS, and with l-arginine (116 mg/liter), l-asparagine (36 mg/liter), l-glutamine (216 mg/liter), streptomycin (0.1 mg/ml) and penicillin (200 U/ml). COS-7 and 293-EBNA cells were grown in DMEM. Cell lines BB49-EBV, K562, and LG-2-EBV were grown in Iscove’s medium. Lines BB49-SCHN and BB49-SCHN.B7 were grown in Iscove’s medium supplemented with ACL-4 (20). All lines were grown in Iscove’s medium supplemented with 10% HS and 8% CO2. The cultures were maintained at 37°C and in 8% CO2.

Stimulation of CTL. Blood mononuclear cells (PBMC) from patient BB49 were isolated by density-gradient centrifugation on lymphoprep (Nycomed Pharma AS, Oslo, Norway) and cryopreserved at –80°C in Iscove’s medium supplemented with 10% human serum (HS) and 10% DMSO. We have verified by DNA fingerprinting that the BB49-SCHN line was of the same genetic origin as the PBMC (21).

Autologous mixed lymphocyte–tumor cell culture (MLTC) was performed as follows: 2 × 10^6 PBMC were stimulated with 10^6 irradiated (100 Gray from a cesium source) BB49-SCHN.B7 cells in 2 ml of Iscove’s medium supplemented with 10% HS and with asparagine, glutamine, and arginine as indicated above (hereafter referred to as complete Iscove’s medium). 25 U/ml of IL-2 and 5 U/ml of IL-4. The CTL clones were cloned by limiting dilution. Each responder cell was cloned by limiting dilution. Each pool of the cDNA library and 50 ng of plasmid pcDNA3 containing an HLA-B3501 cDNA obtained from the RNA of patient LB1047. Isolation of a cDNA Coding for the Autologous HLA-B35 Molecule. Using RT-PCR, we amplified the HLA-B*3503 coding sequence from RNA of EBV-transformed B cells of patient BB49. Primers 5'-CGG GAT CCG CCG AGA TGC GGG 9 and 5'-ACT GCC CGA ATT CTC TCA GTC CCT CAC AAG GCA GCT GTC-3, which are able to bind to most HLA-B sequences, were used as sense and antisense primers. The PCR was carried out for 35 cycles of 1 min at 94°C, 5 s at 59°C, and 5 min at 75°C, using the Pfu DNA polymerase (Stratagene GmbH, Heidelberg, Germany). The PCR product was then cloned into expression vector pcDNA3 (Invitrogen BV) and the sequence was verified.

DNA Sequence Analysis. The double-stranded plasmid template was sequenced on the sense and antisense strands by the dyeoxy-chain termination method (Thermosequenase™ cycle sequencing kit; Amersham, Buckinghamshire, UK). The computer search for sequence homology was performed using the program that is available at blast@ncbi.nlm.nih.gov. Sequence alignments were performed with Geneworks™ software (Intelligenetics, Mountain View, CA).

Production of Progressive Deletions in cDNA 668. To generate progressive deletions from the 3' end of cDNA 668, and thereby obtain a large number of truncated cDNA clones, we used the Erase-7-PCR (Invitrogen Corp., San Diego, CA) reaction. The PCR products were extracted from agarose gels, cloned, and the resulting plasmid DNA was sequenced. The sequence was verified.

Lysis Test and TNF Assay. The BB49-SCHN target cells used in the 51Cr release assay were pretreated for 72 h with 100 U/ml of IFN-γ, and the test was performed as previously described (22). Target cells were also tested for their ability to stimulate the production of TNF by the CTL. In brief, 1,500 CTL were added in flat-bottomed microwells containing 10^4 target cells, in a total volume of 100 μl of Iscove’s complete medium containing 25 U/ml of IL-2. After 4 or 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on cells of WEHI-164 clone 13 (23) in a MTT colorimetric assay (24, 25). Inhibition with mAbs W6/32 (anti-HLA class I), B1.23.2 (anti-HLA-BC), BB7-2 (anti-HLA-A2), or 2B6 (anti-HLA-DR) was performed by addition of a 1/20 dilution of ascites during the experiment.

Construction and Transfection of the cDNA Library. Poly(A)+ RNA was extracted from BB49-SCHN cells using the FastTrack mRNA extraction kit (Invitrogen Corporation, San Diego, CA). mRNA was converted to cDNA with the Superscript Choice System (GIBCO BRL, Gaithersburg, MD) using an oligo-dT primer containing a NotI site at its 5' end. cDNA were then ligated to BstXI adaptors and digested with NotI. After size fractionation, the cDNA were unidirectionally cloned into the BstXI and NotI sites of plasmid pcDNAI/Amp (Invitrogen BV, Leek, The Netherlands). Recombinant plasmids were electroporated into Escherichia coli DH5α F+ and the transfectants were selected with 50 μg/ml of ampicillin. The recombinant library was divided into pools of about 100 cDNA clones. Each pool of bacteria was amplified to saturation and plasmid DNA was extracted using the QIAprep 8 plasmid kit (QIagen GmbH, Hilden, Germany).

Transfection experiments were performed by the DEAE–dextran–chloroquine method (26, 27). In brief, 1.5 × 10^5 COS-7 cells were transfected with about 100 ng of plasmid DNA of a pool of the cDNA library and 50 ng of plasmid pcDNA3 containing an HLA-B*3501 cDNA obtained from the RNA of patient LB1047.

Plate assay was performed as follows: 2 × 10^6 PBMC were stimulated with 10^6 irradiated (100 Gray from a cesium source) BB49-SCHN.B7 cells in 2 ml of Iscove’s medium supplemented with 10% HS and with asparagine, glutamine, and arginine as indicated above (hereafter referred to as complete Iscove’s medium). 25 U/ml of IL-2 and 5 U/ml of IL-4. The responder cells were separated into CD8+ and CD8- in brief, the cells were labeled with anti-human CD8 mAb coupled to magnetic microbeads (Miltenyi Biotec GmbH, Germany). CD8+ cells were separated by passage through a MiniMACS separation column. All 10^6 CD8+ lymphocytes were then restimulated with 5 × 10^6 irradiated BB49-SCHN.B7 cells, in the presence of 25 U/ml of IL-2. On day 21, the responder cells were separated by limiting dilution. Each microwell contained 6 × 10^4 irradiated tumor cells, 2 × 10^4 irradiated LG-2-EBV cells, and 7,500 irradiated CD8- cells as feeder cells in 100 μl of complete Iscove’s medium supplemented with 50 U/ml of IL-2 and 5 U/ml of IL-4. The CTL clones were maintained by passage weekly 5 × 10^4 CTL with 10^6 irradiated BB49-SCHN.B7 cells pretreated for 72 h with 100 U/ml of IFN-γ, in 2 ml of complete Iscove’s medium containing 50 U/ml of IL-2, 0.8 × 10^4 irradiated LG-2-EBV cells and 1.5 × 10^6 allogeneic irradiated PBMC.

Lysis Test and TNF Assay. The BB49-SCHN target cells used in the 51Cr release assay were pretreated for 72 h with 100 U/ml of IFN-γ and the test was performed as previously described (22). Target cells were also tested for their ability to stimulate the production of TNF by the CTL. In brief, 1,500 CTL were added in flat-bottomed microwells containing 10^4 target cells, in a total volume of 100 μl of Iscove’s complete medium containing 25 U/ml of IL-2. After 4 or 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on cells of WEHI-164 clone 13 (23) in a MTT colorimetric assay (24, 25). Inhibition with mAbs W6/32 (anti-HLA class I), B1.23.2 (anti-HLA-BC), BB7-2 (anti-HLA-A2), or 2B6 (anti-HLA-DR) was performed by addition of a 1/20 dilution of ascites during the experiment.
Peptides were tested in chromium release assays where 1,000 $^{35}$Cr-labeled target cells were incubated for 30 min at room temperature in 96-well microplates with various concentrations of peptide, before adding an equal volume containing 10,000 CTL 121 and 50,000 unlabeled K562. The indicated concentrations of peptide represent the final concentrations during the incubation of the target cells with the CTL. The assay was terminated after 4 h of incubation at 37°C.

Site-directed Mutagenesis. In vitro site-directed mutagenesis was performed on cDNA 668 cloned into expression vector pcDNA1/Amp with the Chameleon Kit (Stratagene GmbH, Germany) using the selection primer 5'-TTT GTC TTC CCT TCT GAT TGA TGG TGC TAT TTT G-3'. The coding sequence of mutant cDNA clone 668-wt was verified.

Cell Death Assays. In each microwell, 50,000 293-EBNA cells were transiently transfected with 1 $\mu$l of lipofectAMINE (GIBCO BRL), 40 ng of pcDNA3-β-galactosidase, and 120 ng of the pcDNA1/Amp constructs containing either cDNA clone 668 or 668-wt in a final volume of 50 $\mu$l of OPTIMEM (GIBCO BRL). The experiment was performed in triplicate in two different microplates. After 18 h, cells were fixed in one plate and stained with X-gal (31). The data shown are the percentage of blue apoptotic cells among the 300 blue cells that were counted. In the other plate, the extent of cell death was counted using the Cell Death Detection Plus ELISA kit (Boehringer Mannheim; Indianapolis, IN). 20 $\mu$l of the supernatant of the cell culture (one tenth of total volume) was analyzed. Nucleosomes that are present in the supernatant of the transfected cells are purified by a histone-specific antibody and detected photometrically with an anti-DNA antibody coupled to peroxidase.

**Results**

A CTL Clone Against a Head and Neck Carcinoma. Cell line BB49-SCCHN (referred to below as CHN) was derived from a large squamous cell carcinoma resected from the oral cavity of patient BB49. After irradiation, these cells were used to stimulate either blood mononuclear cells or purified CD8$^+$ T cells collected from patient BB49, but no lytic responder T cells were produced. To improve their stimulatory capacity, CHN cells were transfected with a cDNA encoding costimulator molecule B7-1. The transfectants were cloned by limiting dilution, and clonal line CHN.B7 was selected for further work on the basis of its high expression of B7-1.

Irradiated cells were used to stimulate autologous blood mononuclear cells. After restimulation with the CHN.B7 cells on days 7 and 15, the responder population was found on day 21 to exert low lytic activity on the CHN cells. The CD8$^+$ T cells, which represented less than 5% of the responder population, were sorted and restimulated with CHN.B7. 1 wk later, the responder population efficiently lysed the CHN cells. It was cloned by limiting dilution, and the stable CD8$^+$ CTL clone 121 was obtained. The CTL lysed the autologous tumor cells but did not lyse an autologous EBV-transformed B cell line (BB49-EBV) or autologous PHA-treated blood lymphocytes (Fig. 1).

Patient BB49 was typed HLA-A2, -A11, -B35, -B62, -Cw01, -Cw04. CTL clone 121 secreted TNF upon stimulation with the autologous tumor cells, and TNF release was inhibited by a mAb recognizing HLA-B and HLA-C molecules. This indicates that the antigen recognized by CTL 121 is presented by HLA-B and HLA-C.

A cDNA Coding for the Antigen Recognized by CTL 121. To identify the gene coding for the antigen, we used a genetic approach involving the transfection of a cDNA library into simian cell line COS-7, together with the sequence coding for the HLA presenting molecule (26, 27). In this method, the cDNA library is prepared in an expression vector that contains an origin of replication of SV40, resulting in considerable episomal multiplication of the transfected plasmids in COS-7 cells and therefore in high expression of transfected genes. The transfected COS-7 cells that have integrated the sequence coding for the antigen are then identified on the basis of their ability to stimulate the production of TNF by the CTL.

Not knowing which of the B35, B62, Cw01, and Cw04 was the HLA presenting molecule, we started the screening
of the library with a cDNA encoding HLA-B35 obtained from the RNA of patient LB1047. The cDNA library was prepared with RNA from the CHN cells into expression vector pcDNAI/Amp. The library was divided into pools of 100 recombinant clones and DNA from each pool was cotransfected into microcultures of COS-7 cells. The production of TNF was measured after 24 h of coculture by testing the toxicity of the supernatants for the TNF-sensitive WEHI-164.13 cells. COS-7 cells, cotransfected with HLA-B35 and nonrelevant cDNA clone F10, were used as a negative control. COS-7 cells, cotransfected with HLA-B35 and nonrelevant cDNA clone F10, were used as a negative control. COS-7 cells, cotransfected with HLA-B35 and nonrelevant cDNA clone F10, were used as a negative control.

Figure 3. Stimulation of CTL 121 by COS-7 cells transiently cotransfected with cDNA clone 668 and with a sequence encoding an HLA-B35 molecule. 1,500 CTL were added into microwells containing 10,000 BB49-SCCHN or 20,000 transfected COS-7 cells. The production of TNF was measured after 24 h of coculture by testing the toxicity of the supernatants for the TNF-sensitive WEHI-164.13 cells. (A) COS-7 cells were cotransfected with HLA-B35 and nonrelevant cDNA clone F10, were used as a negative control. (B) COS-7 cells were cotransfected with HLA-B35 and nonrelevant cDNA clone F10, were used as a negative control. COS-7 cells, cotransfected with HLA-B35 and nonrelevant cDNA clone F10, were used as a negative control.

Identification of the Antigenic Peptide. cDNA 668 is 2647 bp long. To identify the region encoding the peptide presented to CTL 121, exonuclease III was used to generate progressive deletions from the 3' end of the cDNA. A large number of truncated cDNA clones were cotransfected into COS-7 cells to test their ability to code for the antigen. Sequence comparison of the longest negative cDNA clone (nucleotides 1–1446) with the shortest positive clone (nucleotides 1–1446) indicated that the COOH-terminus of the antigenic peptide had to be encoded between nucleotides 1446 and 1600. In this region of the putative protein, we searched for peptides bearing the HLA-B35 binding motif, i.e., Pro in position 2, and Tyr or hydrophobic residues at the COOH-terminus of the peptide (33, 34). Two peptides corresponding to this motif were identified, namely nonapeptide FPSD-SWCYF (amino acids 476–484) and octapeptide FPSD-SWCY (amino acids 476–483). They were synthesized and

Figure 4. Lysis by CTL 121 of autologous EBV-transformed B cell line (BB49-EBV) incubated with synthetic peptide FPSDSWCYF. 1000 chromium-labeled BB49-EBV cells were incubated at room temperature for 30 min with the indicated peptides at various concentrations, before adding an equal volume containing 10,000 CTL 121 and 50,000 unlabeled K562. Chromium release was measured after 4 h.
tested for recognition. Only nonapeptide FPSDSWCYF sensitized BB49-EBV to lysis by CTL 121. Half-maximal lysis was obtained with only 3 nM of peptide (Fig. 4).

It is worthy to note that, in addition to CTL 121, we have derived from another blood sample of patient BB49 a second CTL clone recognizing the same antigenic peptide. These two clones express the same TCRBV14S1 and the sequence of the joining region is identical (data not shown).

The Antigenic Peptide Is Encoded by a Mutated CASP-8 Gene.

Recently, two groups reported the identification of a new gene named FLICE or MACH (35, 36). This new gene was more recently renamed CASP-8 and the encoded protein, caspase-8 (37). Caspase-8 is required for induction of apoptosis through the Fas and TNFR1 receptors. cDNA clone 668 is almost identical to the coding sequence of FLICE or the α1 splice variant of MACH (α1). In the region encoding the antigenic peptide, we found a cytosine (C) at position 1508 in cDNA 668 instead of the guanine (G) present in both FLICE and MACH α1 (Fig. 5). This led us to consider the possibility that a tumor-specific point mutation in this gene might be responsible for the expression of the antigen recognized by CTL 121. To compare the CASP-8 sequences in tumor cells and in normal cells of patient BB49, a DNA fragment corresponding to nucleotides 683–1,530 in cDNA 668 was amplified by PCR from genomic DNA of tumor cell line CHN, autologous PHA-treated blood lymphocytes, or BB49-EBV. Direct sequencing of the PCR products revealed that tumor DNA contained both a G and a C at position 1508, whereas DNA from both types of blood cells contained only a G (Fig. 6)

Figure 5. Sequence of the open reading frame and of the putative protein encoded by cDNA 668. The nucleotides are numbered starting from nucleotide 70 of the cDNA 668. The translated Alu sequences are underlined. The sequence corresponding to the peptide recognized by CTL 121 is boxed. For MACH α1 and FLICE, only the stop codon TGA and the differences in nucleotides are indicated. There are two single nucleotide differences with FLICE (This sequence data is available from EMBL/GenBank/DDBJ under accession number U58143), giving rise to two different amino acids, and a single conservative difference with MACH α1 (This sequence data is available from EMBL/GenBank/DDBJ under accession number X98172). The 5′-untranslated region of cDNA 668 is identical to that of MACH α2. Therefore, compared with the cDNA sequences of CASP-8 already described, cDNA 668 is representative of a new type of transcript.

Figure 6. Autoradiography showing the sequence ladder in the region coding for the antigenic peptide in the squamous cell carcinoma line of patient BB49 (BB49-SCCHN), autologous PHA-treated blood lymphocytes (BB49-PBL), autologous EBV-transformed B cell line (BB49-EBV), and the tumor sample resected from patient BB49. (A) PCR products were obtained by amplification with specific primers on genomic DNA. (B) The RT-PCR product was obtained by amplification with specific primers on RNA extracted from the tumor mass resected from patient BB49. The PCR fragments were purified and directly sequenced. The arrow indicates the mutation G to C in one of the alleles.
A tumor antigen described here is encoded by a mutated CASP-8 gene, which codes for protein caspase-8, a key component in the pathway of Fas and TNFR1-induced apoptosis (35, 36). Activation of Fas recruits FADD, the Fas-associated death domain protein, which in turn binds and activates caspase-8 (35, 36, 38, 39). Similarly, TNFR1 recruits TRADD, the TNF R1-associated death domain protein (40). TRADD subsequently acts as an adaptor protein to recruit FADD, which, in a manner that remains to be determined, binds and activates caspase-8. It is believed that the activated caspase-8 activates other caspases, thereby leading to apoptosis.

Caspases are proteases that bear a cysteine in their active site and cleave after an aspartic acid (37). They are synthesized as inactive proenzymes that are cleaved at specific aspartate residues to form active subunits. In the case of caspase-1, the NH₂-terminal prodomain is removed and the other part of the protein is cleaved into a p20 and a p10 subunit. These subunits form heterodimers and their crystal structure has revealed that the mature, active form of caspase-1 is a tetramer composed of two heterodimers (41). Caspase-8 can be cleaved in vitro by granzyme B into an active form containing p20 and p10 subunits (35).

The tumor cells of patient BB49 express both a normal and a mutated allele of CASP-8. The mutated allele codes for a protein with 88 extra residues at the COOH-terminus. Therefore, instead of producing a p10 subunit with 105 amino acids, this altered protein ought to produce a p19 subunit of 193 amino acids. Assuming that caspase-8 adopts a conformation similar to the p20/p10 tetramer of caspase-1, the mutation may decrease the activity of the enzyme because the active tetramer cannot be assembled with the abnormal p19 subunits. Moreover, the p19 subunit may impede the folding of the tetramers containing subunits of the normal caspase-8, thereby resulting in a partially dominant effect.

We have tried to obtain evidence that the CASP-8 mutation effectivly impairs the signaling leading to apoptosis after Fas ligation. When incubated with an anti-Fas antibody, two EBV-transformed B cell lines and the Jurkat cell
line died, as expected. In contrast, the CHN cells did not die, as expected if the mutation impairs apoptosis (data not shown). However, this evidence is not conclusive, because the CHN cells express a lower level of Fas than the EBV-B cells and the Jurkat cells, and we observed that another cell line, expressing the same low level of Fas but not the CASP-8 mutation, did not undergo apoptosis upon treatment with an anti-Fas antibody.

More conclusive evidence for the impairment of the function of the altered caspase-8 was obtained when the mutated CASP-8 gene was transiently transfected in 293-EBNA cells: its ability to trigger apoptosis was reduced relative to the normal caspase-8 but not abolished. This reduction may be more profound in the tumor cells of patient BB49 than in the transient transfectants. Indeed, in the latter, the reduced effectiveness of the altered protein may be partially compensated by a significant overexpression.

Impaired triggering of apoptosis induced by Fas ligand (Fasl) or TNF may provide tumor cells with increased resistance to killing by the immune cells. Clearly, the fact that CHN tumor cells were lysed by CTL 121 indicates that they were not completely protected against an immune attack. This is not surprising, because CTL are known to mediate cytotoxicity not only via Fas but also via a perforin/granzyme-based pathway (42–44). The latter mechanism may not require signaling through caspase-8, because granzyme B is capable of activating other caspases by itself (45, 46). On the other hand, cytolytic T cells are not the only cells that express Fasl. Neutrophils, which express both membrane-bound and soluble Fasl, have been shown to infiltrate tumors and they may kill tumor cells through Fas (47).

The possibility cannot be excluded that other unknown signals leading to apoptosis are also transduced through caspase-8 and that a partial or complete failure to transmit these signals may have offered a selective advantage to the tumor cells of patient BB49. In this regard, another death domain–containing receptor, named DR3, has recently been identified (48). DR3 is related to TNF receptor 1 and Fas, its intracellular signal is transmitted through caspase-8, and its ligand is still unknown.

Using PCR, we screened 150 tumor samples for the presence of a mutation in the stop codon of CASP-8. None was found, suggesting at first that this mutation does not have a very important selective value for tumors. However, it is likely that the mutation in the stop codon is not the only one to be deleterious to the activity of caspase-8. Mutations in other critical parts of the protein, such as the different cleavage sites, the catalytic site, or the domain that interacts with FADD, may be observed in other tumors.

Several tumor antigens recognized on melanoma by autologous CTL also result from point mutations, such as those found in MUM-1, cyclin-dependent kinase 4 (CDK4), and β-catenin (8, 10, 11). The CDK4 and β-catenin mutations have been found in different patients and are clearly both antigenic and oncogenic (10, 49). The CDK4 mutation prevents the protein from binding to its inhibitor, p16. This appears to alter the regulation of the cell cycle, favoring uncontrolled growth of the tumor cells. The β-catenin mutation results in stabilization of the protein, with high levels of free β-catenin driving formation of complexes with transcription factors such as Lef-1. Constitutive β-catenin–Lef-1 complexes may result in persistent transactivation of as yet unidentified target genes, thereby stimulating cell proliferation or inhibiting apoptosis (50). Our results suggest that the CASP-8 mutation may also be oncogenic. It seems likely that other antigens resulting from mutations will be identified with CTL. Some of these mutations may be oncogenic. If the coding genes are unknown, it may well be worth studying their function.

The point mutations of MUM-1, CDK4, and β-catenin change one amino acid and this single substitution generates the antigenic peptide. CASP-8 is interesting in this respect because the last five amino acids of the antigenic peptide recognized by CTL clone 121 are encoded by an extension of the reading frame. The altered caspase-8 comprises 88 extra amino acids that could contain several epitopes presented to other CTL by various HLA molecules.

After the identification of the first tumor antigens recognized by CTL on melanoma cells, concern was expressed that tumors of other histological types may be less likely to express such antigens. The results obtained with renal carcinoma (7, 9) and those reported here for a head and neck carcinoma suggest, however, that there is no fundamental difference in this respect between melanoma and other tumors.
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