Generation of Survivin-specific CD8\(^+\) T Effector Cells by Dendritic Cells Pulsed with Protein or Selected Peptides

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ABSTRACT

The identification of tumor-associated antigens recognized by CD8\(^+\) cytotoxic T cells paved the way to new concepts in adjuvant anticancer therapy. However, the number of tumor-associated proteins found to be expressed in the majority of human cancers is still rather limited. Recently, the newly identified apoptosis inhibitor protein survivin has been recognized as a widely occurring tumor-associated protein. In the present study, we demonstrate that survivin is capable of inducing specific CD8\(^+\) effector T cells in vitro. T cells from healthy donors were subjected to several cycles of stimulation by autologous dendritic cells (DCs) pulsed with soluble recombinant survivin protein. Activation of CD8\(^+\) cytotoxic T cells by survivin-derived peptides cross-presented by DCs was demonstrated by lysis of autologous survivin-expressing B cell transfectants. Using a peptide-motif scoring system, two survivin peptides (ELTGLFLKL and TLPPAWQPFL) were predicted and proved to bind to the HLA-A*0201 molecule. Both peptides were shown to induce CD8\(^+\) effector T cells when presented on DCs; one peptide could be verified to result from natural intracellular processing of survivin. These findings recommend survivin as a new and widely applicable target for protein- and peptide-based immunotherapy of tumors.

INTRODUCTION

In recent years, immunotherapy of human tumors has gained much impetus by the finding that CD8\(^+\) CTLs are capable of recognizing and destroying tumor cells that express peptides that are derived from TAAs and are bound to MHC class I molecules. In addition, clinical studies have shown that the adoptive transfer of tumor-specific CD8\(^+\) CTLs can induce regression of established tumors in melanoma patients. There is good evidence that also tumor-specific CD4\(^+\) T cells are involved in tumor-directed immune reactions. Tumor-specific activation of T cells depends on adequate presentation of tumor-associated peptides. The most effective way to present antigenic peptides seems to be the use of DCs, which are known to be crucial for the initiation of primary T-cell responses. DCs stand out by their capacity to present peptides derived from natural intracellular processing of proteins. The number of well characterized TAAs has steadily increased during the last years and has led to their classification into different groups. The number of relevant TAAs is steadily increasing.

MATERIALS AND METHODS

Cloning of Survivin cDNA. Total RNA was extracted from Jurkat cells using the guanidinium isothiocyanate method. Survivin cDNA of the coding region was generated by RT-PCR using the survivin reverse primer 5'-GAGAGAAAGCTGTCGTTGCTGAGGAACCTGTCGCTGTTG-3' and the survivin forward primer 5'-GAGAGAGATGACCGGTGGTTGCAGTGGTCCCAAGGCTG-3' (both from Life Technologies, Inc., Karlsruhe, Germany), which contain HindIII and BamHI restriction sites, respectively, to facilitate directional cloning. The reverse transcriptase reaction was performed with the first-strand cDNA synthesis kit (Clontech, Heidelberg, Germany) according to the manufacturer’s advice. PCR amplification was carried out in the presence of 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl\(_2\), 50 mM KCl, 0.4 mM dNTP, and 5 units of Taq DNA Polymerase (Amersham Pharmacia Biotech, Braun- schweig, Germany) for 35 cycles in a Bioterma Uno II thermocycler with denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min. The generated PCR product was ligated into the corresponding restriction sites of pBluescript KS, and the correct sequence was verified by sequencing the cloned PCR product on both strands using the ALFexpress Auto Read sequencing kit (Amersham Pharmacia Biotech) with cyanine-labeled universal and reverse primers.

Overexpression and Purification of Recombinant His-tagged Survivin. Survivin cDNA was subcloned into the BamHI and HindIII restriction sites of the procarboxylic expression vector pQE30 (Qiagen, Hilden, Germany), which allows the expression of recombinant proteins with a NH\(_2\)-terminal 6×His-tag. The correct sequence was checked using the ALF-express Auto Read sequencing kit with cyanine-labeled dATP and pQE30-specific primers. Overexpression was performed in Escherichia coli M15[pREP4] (Qiagen), as recommended by the manufacturer. A control culture was grown under the same conditions without induction by isopropyl-1-thio-β-D-galactopyranoside.

Received 2/14/00; accepted 7/5/00.

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1 Supported by Grant 99/009.1 from the Wilhelm Sander-Stiftung (to M. S. and E. P. R.) and by the Medical Faculty, Technical University, Dresden (to M. S. and P. D.).
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4 The abbreviations used are: TAA, tumor-associated antigen; DC, dendritic cell; EGFP, enhanced green fluorescent protein; EBV-BLC, EBV-B lymphoblastoid cell line; IL, interleukin; FACS, fluorescence-activated cell sorting.

CANCER RESEARCH 60, 4845–4849, September 1, 2000
Cells were harvested by centrifugation at 2000 x g and 4°C for 10 min and resuspended in 7.5 ml of lysis buffer [8 M urea, 100 mM Na2HPO4, 10 mM Tris (pH 7.8), 0.1% Triton X-100, and 25 mM imidazole]. After sonication, cellular debris was pelleted at 13,000 x g for 30 min. Purification was carried on by mixing the cleared lysate with 2 ml of the equilibrated Ni-NTA resin (Qiagen) on a rotator for 1 h, followed by washing four times with lysis buffer and eluting with the appropriate buffer containing 100 mM imidazole. The Histagged survivin was refolded by stepwise dialysis against RPMI 1640 (Biochrom, Berlin, Germany). The protein yield was determined by the Bradford assay (Bio-Rad Laboratories, Munich, Germany). The lysates and eluates from both the expression and control culture were analyzed by SDS-PAGE, followed by Coomassie Blue staining or Western blotting. Western blotting was performed according to standard protocols using anti-His antibody (mouse) as primary and alkaline phosphatase-conjugated antioimmunoantibody (mouse) as secondary antibody.

**Transient Transfection of EBV-transformed B Lymphocytes with Survivin.** The coding region of survivin cDNA was amplified by PCR using the forward primer 5’-GAGAGAGGATCCTCAATCCATG-9’ and the reverse primer 5’-GAGAGAGAATTCACAACCATGGGTGCCCCGACG-9’. The amplified product was digested with restriction enzymes and cloned into the expression vector pIRE2-EGFP (Clontech). This permits both survivin and EGFP to be synthesized from a single bicistronic mRNA.

**B Lymphocytes were transfected by supernatant of the EBV-producing cell line B95–8 (kindly provided by Dr. J. Endl (Roche, Penzberg, Germany)). EBV-BLCLs were transfected with pIRE2-survivin/EGFP or pIRE2-EGFP by electroporation. Cell suspension (400 μl; 5 x 10⁵ cells/ml) was transfected with 50 μg of supercoiled plasmid DNA at 280 V/1050 μF (Easyjet T Plus; Equibio, Kent, United Kingdom) and then cultivated in RPMI 1640 supplemented with 10% FCS (both from Biochrom). Eighteen to 24 h after transfection, cells expressing EGFP were selected by FACs and used as target cells.

**Epitope Prediction.** Epitope prediction was done as described (24).

Briefly, potential HLA-A*0201 ligands from the sequence of survivin were selected using a matrix pattern suitable for the calculation of nonamer or decamer peptides fitting to the HLA-A*0201 motif. Such motif predictions are available on our web page.5

**Peptides.** Peptides were synthesized in an automated peptide synthesizer 432A (Applied Biosystems, Weiterstadt, Germany) following the Fmoc/tBu strategy. After removal from the resin by treatment with trifluoroacetic acid/phenoethanedithiol/thioanisole/water (90:3.75:1.25:2.5:2.5 by vol) for 1 h or 3 h, arginine-containing peptides were precipitated from methyl-tert. butyl ether, washed once with methyl-tert. butyl ether and twice with diethyl ether, and resuspended in water prior to lyophilization. Synthesis products were analyzed by high-performance liquid chromatography (system gold; Beckman Instruments, Munich, Germany) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (G2025A; Applied Biosystems, Weiterstadt, Germany) following the Fmoc/tBu strategy. Predicted peptides, each at a concentration of 50 μg/ml, were digested for 4 h with individual peptides at a concentration of 50 μg/ml, washed three times, and then used as target. Chromium-labeled target cells were washed three times and plated in round-bottomed 96-well plates at 5 x 10⁵ cells/well. Eflactor cells were added as triplicates at various E:T ratios. After 4 h of incubation, 100 μl of supernatant was collected from each well and the released ⁵¹Cr was determined in a β-plate scintillator (Wallac, Freiburg, Germany). Maximal and spontaneous release were measured by treating labeled cells with 1% NP40 or medium alone, respectively. The specific cytotoxicity was calculated according to the formula:

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\text{Cytotoxicity} = \frac{(\text{cpm experimental release} - \text{cpm spontaneous release})}{(\text{cpm maximal release} - \text{cpm spontaneous release})} \times 100
\]

**RESULTS**

**Generation of Survivin-reactive CD⁸⁺ CTLs from Blood of Healthy Donors.** To explore whether survivin-specific CTLs can be generated in vitro, CD⁸⁺ T cells were enriched from peripheral blood mononuclear cells of healthy donors and subjected to stimulation with survivin-pulsed DCs. To this end, recombinant His-tagged survivin protein was prepared. After separation on SDS-PAGE, purified survivin appeared as a single band at ~20 kDa, as detected by Coomassie Blue staining and by Western blotting using anti-His antibody (Fig. 1). In this experimental setting, DCs exposed to survivin were supposed to take up and process the protein for cross-presentation as MHC class I-bound peptides. To prove that CTLs were raised against survivin peptides resulting from intracellular processing and not against contaminating peptides found in the survivin preparation, we had to choose target cells expressing peptides that are bona fide derived from survivin by intracellular processing. To verify survivin...
specificity of activated CTLs, survivin-negative homologous target cells had to be included. To fulfill these requirements, we decided to transfect autologous EBV-BLCLs with the cDNA for survivin because in nontransfected EBV-BLCLs survivin-mRNA was not detectable by PCR (data not shown). To evaluate the transfection efficiency and to enrich the survivin-expressing cells, cDNA coding for EGFP was included by using the bicistronic vector pIRES2-survivin/EGFP. Transfection efficiencies were between 10% and 20%, as determined by flow cytometry (Fig. 2A), and survivin-positive target cells could be enriched to a purity of 80–90% by FACS (Fig. 2B). EBV-BLCLs transfected with the control vector pIRES2-EGFP were treated in the same way, resulting in similar transfection and sorting efficiencies (Fig. 2, C and D). The transfected cells served as target cells in a chromium release cytotoxicity assay. Fig. 3 shows marked lysis of pIRES2-survivin/EGFP-transfected and enriched EBV-BLCLs by survivin-activated CD8+ CTLs of both donors, whereas only background activity was seen against pIRES2-EGFP-transfected EBV-BLCLs. These results reveal that survivin-specific CTLs can be generated from T-cell populations of healthy donors when cultured with monocyte-derived DCs pulsed with soluble survivin.

Selection of HLA-A*0201-binding Survivin Peptides and Generation of CTL Responses. The sequence of survivin (accession no. U75285) was screened for peptides containing the HLA-A*0201 peptide motif. The five high-scoring peptides LTLGEFLKL and TLPPAWQPFL (both score 23), and ELTLGEFLKL, KVRRAIEQL, and RAIEQLAAM (all score 19) were synthesized and tested in an HLA stabilization assay after loading onto T2 cells. Only the 10mer peptides ELTLGEFLKL and TLPPAWQPFL showed significant stabilization of HLA-A*0201, most probably due to the presence of the optimal HLA-A*0201 anchor residue Leu both at position 2 and at the COOH terminus. The other peptides without optimal anchor residues did not bind (data not shown). To evaluate the capacity of the HLA-A*0201-binding survivin peptides to induce CTL-enriched CD8+ T lymphocytes were activated by autologous DCs loaded with both survivin peptides. Specific cytotoxicity was determined in a chromium release assay with peptide-pulsed T2 cells as targets. As shown in Fig. 4, peptide-stimulated CD8+ T lymphocytes from donor JB efficiently lysed T2 target cells loaded with either peptide ELTLGEFLKL or peptide TLPPAWQPFL. CTLs from donor FS showed marked lysis of T2 cells only when pulsed with peptide ELTLGEFLKL, but failed to recognize peptide TLPPAWQPFL. To determine whether the predicted survivin peptides were endogenously produced, CD8+ T cells were tested against EBV-BLCLs transfected...
with pIRES2-survivin/EGFP or pIRES2-EGFP. As shown in Fig. 5A, responder T cells of both donors efficiently lysed EBV-BLCLs transfected with pIRES2-survivin/EGFP, whereas no cytotoxicity was observed against EBV-BLCLs transfected with pIRES2-EGFP. Because CTLs of both donors recognized the survivin peptide ELTLGEFLKL it can be concluded that at least this peptide results from intracellular processing. Its presentation by HLA-A*0201 molecules is evidenced by partial inhibition of lysis in the presence of anti-HLA-A2 monoclonal antibody MA 2.1. The specific lysis (%) of T2 cells loaded with peptide ELTLGEFLKL by CTLs of donor JB is shown in Fig. 5A. In contrast, CTLs of donor FS failed to recognize survivin peptide-pulsed T2 cells. The lysis of T2 cells pulsed with peptide ELTLGEFLKL by CTLs of donor JB after induction by protein-pulsed DCs confirm the endogenous processing and MHC class I-restricted presentation of this survivin peptide.

DISCUSSION

In the present study, we provide for the first time evidence that the recently described inhibitor of apoptosis protein survivin, which was found to be selectively expressed in various cancer tissues (22, 23), can induce CD8+ T-cell immune responses in vitro when presented by autologous monocyte-derived DCs. After incubation with whole tumor proteins, DCs process the endocytosed proteins to peptides that are bound to MHC class II molecules and are presented on the cell surface for stimulation of CD4+ T helper lymphocytes. In addition to this classical mechanism of antigen presentation, DCs turned out to be capable of presenting exogenous proteins by an alternative pathway leading to peptide presentation on MHC class I molecules, which is referred to as cross-presentation (7, 26). The efficient induction of antigen-specific CTL responses by protein-pulsed DCs was demonstrated by recent in vivo experiments (27, 28). In the present study, DCs incubated with soluble recombinant survivin were shown to induce specific MHC class I-restricted CTLs. To prove that these CTLs were induced by survivin peptides cross-presented by DCs, we chose autologous EBV-BLCLs as targets that had been transfected with cDNA for both survivin and EGFP using a bicistronic vector construct. We reasoned that this approach might be of 2-fold advantage. First, it provided appropriate control target cells because EBV-BLCLs not transfected or only transfected with the EGFP-cDNA did not express survivin. Second, the transiently transfected EBV-BLCLs could be enriched by FACS to obtain a suitable survivin-expressing target cell population. Transient transfection with the bicistronic EGFP-cDNA-containing vector combined with FACS seems to be particularly useful in experiments where high-frequency expression of proteins is attempted without establishing a stably transfected cell line. When assayed against these transfected EBV-BLCL targets, CTLs generated by stimulation with survivin-pulsed DCs performed their cytotoxicity in a survivin-dependent manner. From these results we conclude that monocyte-derived DCs cross-present survivin peptides and are capable to efficiently induce survivin-specific CTLs from lymphocytes of healthy donors.

Fig. 4. Generation of survivin-reactive CTLs by peptide-loaded DCs. CTLs of two healthy donors were activated against the two survivin-derived peptides (P) ELTLGEFLKL and TLPPAWQFPL presented by autologous DCs. T2 cells loaded with either of the two peptides were labeled with 31Cr. T2 cells without survivin-derived peptides served as negative controls. After 4 h of incubation with CTLs, chromium release was measured. Symbols represent the means of triplicate chromium release determinations, SE being <10% in all determinations.

Fig. 5. Recognition of endogenously produced survivin-derived peptides by survivin-reactive CTLs. A, CTLs of two blood donors induced by DCs pulsed with the two predicted survivin peptides were cocultured for 4 h with chromium-labeled EBV-BLCLs transfected with pIRES2-survivin/EGFP or pIRES2-EGFP as control at various E:T ratios. Symbols represent the means of triplicate chromium release determinations, SE being <10% in all determinations. B, inhibition of T cell-mediated cytotoxicity against survivin-expressing EBV-BLCLs at an E:T ratio of 30:1 in the presence of anti-HLA-A2 monoclonal MA 2.1. The columns represent the means of triplicate chromium release determinations. Bars, SE.

Fig. 6. Recognition of survivin peptide-loaded T2 cells by specific CTLs induced with protein-pulsed DCs. Activated CTLs were cocultured with chromium-labeled T2 cells loaded with survivin-derived peptides (P) ELTLGEFLKL or TLPPAWQFPL and assayed against the T2 cells at various E:T cell ratios (3:1, 10:1, 30:1). T2 cells without survivin-derived peptides served as negative controls. After 4 h of incubation, chromium release was measured. Symbols represent the means of triplicate chromium release determinations, SE being <10% in all determinations.

Fig. 5. Recognition of endogenously produced survivin-derived peptides by survivin-reactive CTLs. A, CTLs of two blood donors induced by DCs pulsed with the two predicted survivin peptides were cocultured for 4 h with chromium-labeled EBV-BLCLs transfected with pIRES2-survivin/EGFP or pIRES2-EGFP as control at various E:T ratios. Symbols represent the means of triplicate chromium release determinations, SE being <10% in all determinations. B, inhibition of T cell-mediated cytotoxicity against survivin-expressing EBV-BLCLs at an E:T ratio of 30:1 in the presence of anti-HLA-A2 monoclonal MA 2.1. The columns represent the means of triplicate chromium release determinations. Bars, SE.
A whole array of strategies in experimental immunotherapy of cancer are based on peptide vaccination. Because survivin seems to be an almost universal TAA (22, 23) it seemed an important task to identify relevant HLA class I-restricted survivin peptides. In a first approach we made a search for survivin peptides displaying the HLA A*0201 peptide motif, because HLA A*0201 is the most frequent HLA-A allele in Caucasian individuals. Among five scoring peptides, two peptides (ELTLGEFLKL and TLPPAWQPFL) were identified that showed significant stabilization of HLA A*0201 molecules at the surface of T2 cells. Both peptides proved to be immunogenic because they induced specific CTLs in vitro. The peptide-induced CTLs of both donors included in the experiments were found to lyse T2 cells being too low to induce T-cell activation. Finally, the failure of T cells precursors in the blood or to an overall affinity of the T-cell receptors for survivin-derived peptides, which are presented by various HLA-class I molecules on DCs after processing for cross-presentation.

To further substantiate the survivin peptide ELTLGEFLKL as a potent candidate antigen for immunotherapy of tumors it remains to be shown that it is naturally exposed on tumor cells for recognition by CTLs. Because most prepared tumor tissues (22) and all tumor cell lines (29) seem to express survivin, a survivin-negative target cell that could serve as an appropriate specificity control is missing. For this reason, specific recognition of survivin on tumor cells can only be proven by the use of cloned CTLs that are not yet available.

In summary, we show here for the first time the specific activation of CD8+ CTLs against the widely occurring tumor-associated protein survivin. Thus, survivin recommends itself as a universal antigen for the design of protein- and peptide-based anticancer vaccines.

ACKNOWLEDGMENTS

The technical assistance of Karin Guenther, Baerbel Loebel, and Barbara Uteß is greatly appreciated. We thank Uta Oelschlägel for FACS.

REFERENCES

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*Cancer Res* 2000;60:4845-4849.

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