The Chemotherapeutic Drug 5-Fluorouracil Induces Apoptosis in Mouse Thymocytes in Vivo via Activation of the CD95(APO-1/Fas) System

Sören T. Eichhorst, Susanne Müerköster, Markus A. Weigand, and Peter H. Krammer†

Tumor Immunology Program, German Cancer Research Center (DKFZ) [S. T. E., S. M., M. A. W., P. H. K.] and Clinic of Anesthesiology, University of Heidelberg [M. A. W.], 69120 Heidelberg, Germany

ABSTRACT

The CD95(CD95 ligand (CD95L) system has been shown to mediate chemotherapeutic drug-induced apoptosis in vitro. However, the contribution of the CD95 pathway to drug-induced apoptosis is controversial. We have shown previously that 5-fluorouracil (5-FU) induces apoptosis in vitro via the activation of the CD95/CD95L system. To study the effects of the chemotherapeutic drug 5-FU and the contribution of the CD95 system to 5-FU-induced apoptosis in vivo, we gave mice an i.p. injection of 5-FU. Apoptotic cell death peaked in thymocytes at 18 h after administration of 5-FU. Total organ weight and cell number in the thymus were reduced by approximately 40%. This cell loss was due to apoptosis, as measured in cell suspensions by measuring hypodiploid DNA content and by terminal deoxynucleotidyl transferase-mediated nick end labeling staining of tissue sections. The number of apoptotic cells correlated with the extent of weight loss and cell attrition of the organs. Furthermore, in the thymi of 5-FU-treated animals, CD95L was strongly up-regulated. Apoptosis of thymocytes was blocked in vivo with neutralizing anti-CD95L antibodies. In addition, cell loss in the thymus was negligible in lpr mice in comparison with wild-type mice. Thus, a significant portion of apoptosis of thymocytes in vivo on treatment with 5-FU is mediated via the CD95/CD95L pathway. Our findings therefore contribute to the understanding how chemotherapeutic drugs exert their effects in vivo.

INTRODUCTION

Chemotherapeutic drugs are a major tool in anticancer therapy. However, most of the chemotherapeutic drugs used are nonspecific and cause severe side effects. Thus, a molecular understanding of the mechanism of drug action is desirable to develop a more rational therapy.

Chemotherapeutic drugs can induce apoptosis in tumor cells (1). However, how these drugs induce apoptosis is controversial. One possible mechanism is induction of apoptosis via death receptors, such as CD95(APO-1/Fas) (2). According to this paradigm, chemotherapeutic drugs induce expression of CD95 and CD95L on tumor cells that subsequently die via an autocrine suicidal mechanism or a paracrine mechanism (3–7). Recently, this concept has been questioned due to the fact that cells from knockout mice for caspase 8 or FADD show little resistance to induction of apoptosis on administration of chemotherapeutic drugs in vitro (8, 9). However, in vitro treatment of cells from apaf-1 and caspase 9 knockout mice with chemotherapeutic drugs revealed relative resistance of these cells to the induction of apoptosis (10–12). Furthermore, it was shown that FADD dominant-negative-overexpressing mice are also not protected from the effects of these drugs (13). In contrast, CD95L-neutralizing reagents were shown to effectively block drug-induced apoptosis of tumor cell lines derived from different tissues (3–7). Because these experiments were performed in vitro, and little is known about the in vivo situation, we investigated the role of the CD95 system in vivo. To study the involvement of CD95, we injected the chemotherapeutic drug 5-FU into mice. 5-FU is a widely used chemotherapeutic drug used for treatment of different primary and metastatic malignancies. 5-FU inhibits thymidilate synthase, thereby causing thymineless stress and subsequent DNA and RNA damage. Recently, we have shown that 5-FU causes apoptosis in vitro that is partially mediated via the CD95 system and is caused by up-regulation of CD95 and CD95L (14). We now show that thymocytes and, to a minor extent, splenocytes in mice undergo apoptosis after a single injection of 5-FU. In addition, after 5-FU administration, CD95L was strongly up-regulated on thymocytes and was up-regulated to a lesser extent on splenocytes. Furthermore, the attrition of thymocytes was blocked by administration of a CD95L-antagonistic antibody. The effect of the drug in the thymus was also impaired in lpr mice compared with wild-type mice. Thus, the CD95/CD95L system plays a significant role in the induction of thymocyte apoptosis of mice on chemotherapeutic drug treatment in vivo. These findings contribute to the understanding of how chemotherapeutic drugs exert their effects in vivo.

MATERIALS AND METHODS

Mice

Adult C57BL/6 and C57BL/6lpr (B6 lpr/lpr) mice (age, 6–10 weeks) were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice received i.p. injection of 5 mg of 5-FU (Ribosepharm GmbH, München, Germany), 500 μg of dexamethasone (Sigma, Deisenhofen, Germany) or PBS. For blockade, 100 μg of anti-CD95L antibody or 100 μg of isotype-matched control antibody were administered i.p. concomitantly with the injection of 5-FU.

Antibodies

The neutralizing endotoxin-free antiaouse CD95L antibody MFL-3 (hamster IgG1) and an isotype-matched hamster control antibody were purchased from Pharmingen (Hamburg, Germany). For immunostaining of CD95L, polyclonal goat antiaouse antiserum was used (Santa Cruz Biotechnology Inc., Heidelberg, Germany). As an isotype control for CD95L stainings, a goat IgG antibody was used (Santa Cruz). The blocking peptides were either specific CD95L peptide (Santa Cruz) or irrelevant bcl-xL peptide (German Cancer Research Center; peptide synthesis).

Preparation of Cell Suspensions

Thymi and spleens were mashed between frosted ends of glass slides and passed through a sieve. After washing several times, erythrocytes were lysed in a hypotonic ammonium chloride buffer for 7 min at room temperature. After repeated washing, the cells were cultured in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Life Technologies, Inc.), 100 μg/ml gentamycin (Life Technologies, Inc.), and 50 μM β-mercaptoethanol (Sigma) at 37°C in a 5% CO2/95% humidified air atmosphere.

Flow Cytometry Analyses

To determine cell death, cells were washed twice in PBS and stained with 2.5 μg/ml propidium iodide (Sigma). Uptake of the dye was measured in a
FACScan flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany) using the CellQuest software. Concomitant changes in forward/side scatter of the cells were evaluated.

For quantification of DNA fragmentation, cells were centrifuged at 200×g and washed. They were lysed in a hypotonic lysis buffer (0.1% sodium citrate and 0.1% Triton X-100) containing 50 μg/ml propidium iodide and incubated at 4°C overnight. The nuclei were then analyzed for DNA content by flow cytometry (15).

For surface labeling of CD4 and CD8, C57BL/6 mice were treated with either 5 mg of 5-FU or with 500 μg of dexamethasone i.p. or left untreated. In addition, lpr mice were treated with 5 mg of 5-FU i.p. or left untreated. Animals were sacrificed 18 h after treatment, and the thymi were removed. Three thymi per animal were pooled, and single cell suspensions were prepared. Cells were washed in FACS buffer (PBS supplemented with 5% FCS) and incubated with a FITC-labeled anti-CD8 IgG2a monoclonal antibody and a phycoerythrin-labeled anti-CD4 IgG2a monoclonal antibody (both from Pharmingen). After a 10-min incubation on ice, cells were washed in PBS and resuspended in FACS buffer. Ten thousand events were counted, and the data were expressed as dot plots.

Detection of mRNA Expression by RT-PCR

Thymi were taken out and immediately shock frozen in liquid nitrogen. Whole-organ lysates were prepared in a mortar under liquid nitrogen. RNA from harvested tissue was prepared using the RNeasy Kit (Qiagen GmbH, Hilden, Germany) according to the instructions of the manufacturer. For each isolation, approximately 10 mg of frozen tissue was used. One μg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) with oligo(dT)15 primers (Roche GmbH, Mannheim, Germany) in a 20-μl reaction containing 10 mm DTT and 500 μM deoxynucleotide triphosphates. One-μl aliquots were amplified in a DNA thermocycler (Stratagene, Heidelberg, Germany) with 0.5 unit of Taq DNA polymerase (Roche GmbH, Mannheim, Germany) in a 50-μl reaction. Thirty reaction cycles were performed. Each cycle consisted of a denaturation step (94°C for 30 s), an annealing step (56°C for 30 s), and an elongation step (72°C for 30 s). The reaction was completed with a 72°C elongation step for 10 min. PCR products were analyzed on agarose gels.

Primers were purchased from MWG Biotech GmbH (Ebersberg, Germany). Primer sequences were as follows: mouse CD95L sense (5′-GACAGAAGAGAAAGGATAC-3′) and mouse CD95L antisense (5′-TCTCCATCCATTAGCACCAGATCC-3′); yielding a PCR product of 612 bp; and mouse CD95 sense (5′-GGCAGAAGAGAAAGGATAC-3′) and mouse CD95 antisense (5′-GCTCTGACATCTTTGCGGA-3′), yielding a product of 450 bp. Each reverse transcribed mRNA was internally controlled with a β-actin PCR using the primers mouse sense (5′-ATTGTACACATCTGTGGAGATCT-3′) and antisense (5′-CTTCATGAGTATGTCTGTCATG-3′), yielding a PCR product of 350 bp.

Immunohistochemistry

Tissue Preparation. Thymi from C57BL/6 mice were removed at different time points after 5-FU injection and snap-frozen in liquid nitrogen. Six-μm-thick consecutive cryostat sections were mounted on uncovered glass slides, air-dried, and fixed in acetone (Merck, Darmstadt, Germany) for 10 min at room temperature and air-dried again for 1 h.

CD95L Staining. After drying, slides were washed in PBS three times for 5 min. To avoid nonspecific binding, sections were treated with 2% (w/v) normal mouse serum for 15 min, followed by incubation with a polyclonal serum anti-CD95L (Santa Cruz) for 30 min. After washing three times in PBS, sections were treated with a biotinylated second antibody (Dianova, Hamburg, Germany). After washing again, an incubation with streptavidin conjugated with AP (Sigma) followed for 30 min. Before the substrate reaction for AP was performed, slides were washed three times in PBS for 5 min. After staining, the sections were washed with water, counterstained in 50% (w/v) haemalum (Merck), and mounted with glycerol-gelatine (Merck). The same protocol was performed for negative controls, in which either the first or the second antibody was omitted, or the respective isotype control was used. All steps were performed in a humid chamber and at room temperature. To further control the specificity of the staining, anti-CD95L polyclonal antibody was preincubated with a 5-fold excess of either the specific CD95L peptide or with the irrelevant bc1-x peptide for 2 h at room temperature. These solutions were then used as first antibody reagents. The staining protocol was performed as described.

Development of the AP Substrate Reaction. The substrate for the development of AP consisted of 6.3 μl of 5% (w/v) Neufuchsin (Sigma) in 16 μl of 4% sodium nitrite (Fluka, Buchs, Switzerland), 2 mg of naphthol-AS-Bi-phosphate (Sigma) in 20 μl of N,N-dimethylformamide (Merck), and 3 ml of 0.05 m Tris-HCl buffer, (pH 8.7) containing 1 mM levamisole (Sigma). The freshly prepared solutions were filtered through a 0.22 μm filter (Millipore, Eschborn, Germany) and added to the sections. Development lasted about 3–10 min, with regular checking of the staining intensity using a microscope. Immunohistochemical results were evaluated by counting the number of positively stained cells per thymus or white pulp in the spleen from respective animals. The means and SDs of the data obtained from three mice/time point and from several experiments were calculated and presented in graphs.

TUNEL Assay

To detect apoptotic cells, the TUNEL assay was performed with the in situ TCCACAGCTGTC-3′), and mouse CD95L antisense (5′-TCTCCATCCATTAGCACCAGATCC-3′), yielding a PCR product of 612 bp; and mouse CD95 sense (5′-GGCAGAAGAGAAAGGATAC-3′) and mouse CD95 antisense (5′-GCTCTGACATCTTTGCGGA-3′), yielding a product of 450 bp. Each reverse transcribed mRNA was internally controlled with a β-actin PCR using the primers mouse sense (5′-ATTGTACACATCTGTGGAGATCT-3′) and antisense (5′-CTTCATGAGTATGTCTGTCATG-3′), yielding a PCR product of 350 bp.

RESULTS

To investigate the effect of 5-FU in vivo, C57BL/6 mice received i.p. injection of either 5 mg of 5-FU as a control. On gross examination, there was no difference between treated and untreated mice. In particular, treated mice and untreated mice showed a similar consumption of water (data not shown). Interestingly, as shown in Fig. 1A, different tissues reacted to a different extent to the administration of the drug. Whereas thymi of 5-FU-injected mice showed a reduction of total organ weight by approximately 40%, spleens and livers showed a less pronounced response to the drug. Spleen weight was only reduced by ~15%, and liver weight was reduced by ~5% in comparison with respective organs of untreated mice 18 h after injection. Because the observed effect was most pronounced in the thymus, we concentrated on this organ in additional experiments. Reduction of thymus weight was also reflected at the cellular level because cell numbers were diminished to the same extent as whole organ weight (Fig. 1, B + C). To test whether apoptosis of thymocytes in vivo involves the CD95/CD95L pathway, we coinjected mice i.p. with 5 mg of 5-FU and 100 μg of neutralizing anti-CD95L antibody MFL-3. As shown in the left part of Fig. 1, B and C, coadministration of MFL-3 rescued thymocytes from deletion. In addition, injection of lpr mice with 5-FU and incubation for 18 h did not lead to significant differences in organ weight or cell number of thymus (Fig. 1 B, right part). Taken together, these findings indicate a role for the CD95 pathway in the observed deletion of thymocytes in vivo on chemotherapeutic treatment.

To confirm that the deletion of thymocytes on treatment with 5-FU is due to induction of apoptosis, DNA fragmentation in isolated...
compared with injection with 5-FU and control antibody (Fig. 2). MFL-3 isolated thymocytes showed less hypodiploid DNA content when mice received injection of 5-FU, and the CD95L blocking antibody thymocytes was also reflected on the apoptotic cell level. Thus, when administration of chemotherapeutic drugs is mediated partially via the CD95/CD95L system. The diminished loss of thymocytes was investigated according to Nicoletti et al. (15). In the thymus, the number of cells with subdiploid DNA content was drastically increased in 5-FU-treated mice compared with controls (Fig. 2A). We and others have demonstrated that apoptosis in various cell types in vitro on administration of chemotherapeutic drugs is mediated partially via the CD95/CD95L system. The diminished loss of thymocytes was also reflected on the apoptotic cell level. Thus, when mice received injection of 5-FU, and the CD95L blocking antibody MFL-3 isolated thymocytes showed less hypodiploid DNA content compared with injection with 5-FU and control antibody (Fig. 2A).

This effect was also evident in vivo in TUNEL-labeled tissue sections. The number of apoptotic cells in the thymus in vivo increased early and remained elevated for at least 9 h after 5-FU injection (Fig. 2B). There was a massive loss of total thymocytes distributed equally over all thymocyte subpopulations. Whereas ~50% of thymocytes were depleted, the CD4+/CD8+ thymocyte subpopulation was not preferentially deleted (Fig. 3). Treatment of lpr mice with 5-FU did not lead to a significant thymocyte attrition. When wild-type mice were injected with 500 μg of dexamethasone i.p., apoptosis was induced in ~50% of thymocytes (data not shown). However, in this case, double-positive (CD4+/CD8+) thymocytes were preferentially affected (Fig. 3).

Next, we tested whether transcription of CD95L is involved in this process as has been shown in vitro (3). As displayed in Fig. 4, CD95L mRNA was strongly up-regulated in thymi of 5-FU-treated mice. CD95 was concomitantly expressed in control thymus and was further up-regulated after treatment with 5-FU (Fig. 4). To test whether mRNA up-regulation led to an increase in CD95L protein on thymocytes of 5-FU-treated mice, we established immunohistochemistry for this protein. Expression analysis of CD95L by immunohistochemistry is known to be difficult. Several reports used antisera with questionable specificity to demonstrate CD95L expression. Therefore, we first checked the specificity of CD95L staining on murine thymus (Fig. 5). Although we observed some background staining, especially reflected by staining of blood vessels and connective tissue components, we detected specific staining for CD95L (Fig. 5A) Staining was inhibitable by specific immunogenic peptides (Fig. 5C), but not with unrelated bcl-xL peptides (Fig. 5D). Staining with an isotype-matched control antibody was negative (Fig. 5B). Therefore, we could specifically detect murine CD95L by immunohistochemistry, and we analyzed CD95L expression on thymus sections of 5-FU-treated animals.
cytometry. The thymocyte population was deleted to stained with anti-CD4 and anti-CD8 antibodies, and the cells were analyzed by flow thymocytes was unchanged in lpr mice treated with 5-FU. Live-gated cells were analyzed, treated with 5-FU and wild-type mice treated with dexamethasone. The total number of in situ At the same time, an increased number of apoptotic cells appeared in (Fig. 6 B A CD95L protein was seen already at 2 h after treatment, peaked at 4 h; Furthermore, up-regulation of CD95L protein was rapid because new CD95L was up-regulated in thymi of mice treated for 3 h with 5-FU; CD95L was up-regulated in thymi of mice treated for 3 h with 5-FU. This up-regulation could also be observed at 2 h after treatment, peaked at ~4 h, and remained elevated for at least 9 h (Fig. 7).

In summary, we could show that murine thymocytes undergo apoptosis after a single injection of 5-FU. Apoptosis is mediated, at least in part, by the CD95/CD95L system and involves up-regulation of both proteins on mouse thymocytes.

**DISCUSSION**

We have demonstrated, that a major fraction of thymocytes of 5-FU-treated mice are depleted by CD95-mediated apoptosis. Administration of the drug caused a rapid up-regulation of CD95L in the thymus starting 2 h after injection. Up-regulation could also be observed at this time point in the spleen, although to a smaller extent. Up-regulation of CD95L protein was functionally relevant in the thymus because apoptosis of thymocytes could be blocked by coadministration of a neutralizing anti-CD95L antibody but not control immunoglobulin and was diminished in lpr mice. Our data also indicate that none of the thymocyte subpopulations was a preferential target of 5-FU.

Thus far, it is still controversial whether and to what extent death receptor pathways are involved in chemotherapy-induced apoptosis (8). Data from our group and others indicated that in vitro, CD95 is partially involved in apoptosis by various chemotherapeutic drugs in certain cell types (3–7). Data from several groups support our findings (16, 17), but contradictory data came from the observations that cells from mice that lack essential components of death receptor signaling pathways such as FADD or caspase 8 are only minimally protected from drug-induced apoptosis (8, 9, 13). However, these data are almost exclusively derived from investigations of primary cells in vitro or from mouse embryonic fibroblasts. These findings are therefore not directly comparable with data derived from treatment of tumor cells. In addition, most of these studies were done in vitro. In contrast, our studies reported here examined the role of the CD95/CD95L system in drug-induced apoptosis in vivo. The difference between the in vitro and in vivo findings could be explained in part by the microenvironment and spatial order in which cells are located in the body compared with the situation in the culture dish. CD95 and CD95L might be in close contact in vivo, and therefore apoptosis might be easily transmitted via this pathway. Similar findings were published for induction of activation-induced cell death in T-cell hybridomas and mouse T-cell blasts by Bonfoco et al. (18). This group showed that activation-induced cell death is density dependent, suggesting the requirement of cell-cell contact to induce “fratricide.” Similarly, close cell-cell contact may also be required for drug-induced apoptosis. Moreover, the type of neighboring cells may also influence the outcome of drug treatment. Schwarz et al. (19) showed that follicular dendritic cells, when cocultured with malignant B cells, could protect the tumor cells from apoptosis induced by anti-CD95 antibody and chemotherapeutic drugs. In addition, the lack of decreased sensitivity in CD95 signaling-deficient mice does not exclude the possibility that the CD95 system plays a role in drug-induced apoptosis in mice in which the system is functional. On the other hand, other death systems may also be involved because the attrition of cells in the thymus of lpr mice in our experiments was delayed and diminished but not totally abrogated, and blocking with the anti-CD95L antibody was not complete. Therefore, we conclude that the CD95 system is not absolutely required for chemotherapy-induced apoptosis (20), but it is sufficient to mediate apoptosis after cellular stress as exerted by chemotherapeutic drugs. A similar mechanism has also been described for radiation- and heat shock-induced apoptosis (21). However, if the CD95 pathway is not functional, other potential apoptosis-promoting systems, e.g., those involving bax (22–24), may substitute for it (25). In addition, in vivo, apoptosis is possibly highly tissue specific, i.e., different tissues might react differently with regard to the use of death systems on administration of chemotherapeutic drugs.

Furthermore, in vivo, a differential sensitivity of cells from different tissues to the regulation of CD95L is observed because different
tissues reacted differently and with different kinetics to drug administration. Liver tissue, which was exposed to the highest concentrations of the drug due to the i.p. application route of 5-FU, showed little CD95L up-regulation up to 18 h after treatment (data not shown), whereas the spleens showed intermediate CD95L up-regulation, and the thymi showed the highest CD95L expression at this time point. These data might be due to a different activation of cellular stress pathways. Thus, we have recently shown that CD95L expression on

![Immunohistochemistry for CD95L in murine thymus. A, anti-CD95L antibody staining; B, staining with isotype-matched control antibody; C and D, staining with anti-CD95L antibody, but blockade with specific peptide (C) or unspecific bcl-xL peptide (D). Arrows indicate CD95L-positive cells. 100×.](image)

![Immunohistochemistry for CD95L expression (A and B) and TUNEL staining of apoptotic cells (C and D) in thymus of control (A and C) or 5-FU-treated mice (B and D) 3 h after injection of 5 mg of 5-FU. One representative experiment of three is shown. 100×.](image)
5-FU exposure depends on activation of the MAP/ERK kinase kinase/c-Jun kinase kinase cascade and the transcription factor AP-1 (26). The fact that we did not observe a differential depletion of CD4+/CD8+ thymocytes is in line with findings from other laboratories. Different mouse thymocyte subpopulations do not show differential expression for CD95 as demonstrated for human thymocytes (27). Moreover, thymus sections were stained for CD95L expression by immunohistochemistry. Sections of thymi were prepared at different time points after treatment with 5-FU. Subsequently, thymus sections were prepared for CD95L expression by immunohistochemistry. Positive cells were counted in a double-blinded fashion. Data are the mean with SD from three thymus/time point. Two independent experiments with a similar outcome were performed.

In summary, our investigations of the in vivo role of the CD95/CD95L pathway in drug-induced apoptosis have demonstrated that this system contributes to the attrition of cells on 5-FU treatment, particularly in the thymus. This finding may be of clinical importance for patients treated with 5-FU. Thus, immune suppression in these patients might not only be due to peripheral deletion of lymphocytes but may also be the result of a reduction of lymphocyte precursors in the thymus via CD95/CD95L interactions.

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