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α-Fetoprotein Impairs APC Function and Induces Their Apoptosis

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α-Fetoprotein (AFP) is a tumor-associated Ag, and its serum level is elevated in patients with hepatocellular carcinoma (HCC). In vitro, AFP induces functional impairment of dendritic cells (DCs). This was demonstrated by the down-regulation of CD40 and CD86 molecules and the impairment of allostimulatory function. Also, AFP was found to induce significant apoptosis of DCs, and AFP-treated DCs produced low levels of IL-12 and TNF-α, a cytokine pattern that could hamper an efficient antitumor immune response. Ex vivo, APCs of patients with HCC and high levels of AFP produced lower levels of TNF-α than that of healthy individuals. In conclusion, these results illustrate that AFP induces dysfunction and apoptosis of APCs, thereby offering a mechanism by which HCC escapes immunological control. The Journal of Immunology, 2004, 173: 1772–1778.

Structurally related to human albumin, α-fetoprotein (AFP) is a well-characterized oncofetal Ag. It is normally expressed during embryogenesis and is present in only trace amounts in normal adults (1). However, the expression of the AFP gene is reactivated in patients with testicular and hepatocellular carcinoma (HCC) with high levels of AFP being found in the sera and tumor tissues. The determination of serum AFP aids in the diagnosis and the management of patients with HCC. A serum AFP level of >200 ng/ml is shown to have a specificity of 100% for HCC (2). In a study of 68 Asian-American patients with HCC, serum AFP ranged from 0 to 636,000 ng/ml with the average being 5,200 ng/ml (3). AFP, like serum albumin, shows relatively strong binding affinities for a variety of ligands. Various other specific physiological roles for AFP are being proposed such as its possible role in the regulation of immune cells (1). A series of investigations has provided evidence that AFP causes selective down-regulation of MHC class II on monocytes (4, 5) and the suppression of T (6–9) and B lymphocyte (10) responses. It has also been shown that AFP-mediated immunoregulation is an activity intrinsic to the molecule itself and cannot be attributed to either putative noncovalently bound moieties or to posttranslational modifications such as glycosylation and sialylation (11). Stimulation of leukotriene synthesis by AFP in macrophages has been suggested as a possible mechanism for its immunoregulatory effects (12). In addition, microscopic autoradiography has exhibited binding of AFP almost exclusively on human peripheral monocytes but not on lymphocytes (13), suggesting that the regulatory effects of AFP may be via APCs.

Dendritic cells (DCs) are the most potent APCs of the immune system and are crucial in the initiation of the immune response against pathogens and tumors. DCs exist in two differing states of maturation: immature and mature. Immature DCs express low levels of surface molecules such as CD80, CD86, CD40, and MHC class II, and have low T-cell-stimulatory capacity. Several stimulatory agents, such as proinflammatory cytokines and viral or bacterial products, can trigger DC activation and thus maturation. This is defined by the up-regulation of costimulatory molecules, an increase in the levels of TNF-α and IL-12 production, and improved capacity to stimulate T cells (14, 15). The activated DCs can rapidly activate other innate immune cells such as NK (16) and NKT cells (17). Most knowledge about the biology of DCs has emerged from the ability to generate DCs in vitro from either CD34+ hematopoietic progenitors or peripheral blood monocytes.

In this study, we show that the treatment of monocyte-derived DCs with AFP induces DCs dysfunction as detected by the down-regulation of surface molecules and inhibition of their T cell-stimulatory capacity. In addition, AFP treatment reduces the ability of monocyte-derived DCs to produce TNF-α and IL-12 and induces apoptosis of DCs. Furthermore, we compare the ability of APCs from patients with HCC to produce TNF-α with that of control individuals. The data clearly show that HCC with high levels of serum AFP have reduced TNF-α production.

Materials and Methods

Patients

The patients involved in this study were all reviewed at the Liver Unit of the Cromwell Hospital (London, U.K.). The ethical committee’s approval was granted, and informed consent was obtained. In total, there were 16 patients with an AFP value ranging from 8 to 1,141,205 ng/ml. The average serum AFP level in the HCC group was 80,409 ng/ml. There were seven patients with hepatitis C cirrhosis, two with cryogenic cirrhosis, three with hepatitis B cirrhosis, two with hepatitis B and C cirrhosis, one with alcoholic liver cirrhosis, and one with alcoholic liver and hepatitis C-related cirrhosis. Five of them were Child’s grade B and 11 were Child’s grade A. Laboratory tests including aspartate aminotransferase, alanine aminotransferase, total bilirubin, platelet count, prothrombin time, international normalized ratio, creatinine, hepatitis B surface Ag, anti-hepatitis C virus Ab, and hepatitis C virus RNA, were determined using standard, commercially available assays. All blood samples from patients with HCC
were withdrawn before giving any therapy. The severity of cirrhosis was assessed by Child-Pugh score.

**AFP measurement**

Levels of serum AFP were measured using microparticle enzyme immunoassay (MEIA) kit obtained from Abbott Laboratories (Abbott Park, IL) and performed according to the manufacturer’s instruction. In brief, anti-AFP microparticles were incubated with the blood specimen, and an aliquot of the reaction mixture was transferred to the matrix cell. The matrix cell was washed, removing unbound materials, and the anti-AFP conjugate was dispensed onto the matrix cell. The substrate was added to the matrix cell, and the fluorescent product is measured by the MEIA optical assembly.

**Cell culture**

RPMI 1640 medium, penicillin and streptomycin, and 10% heat-inactivated FCS were purchased from Invitrogen Life Technologies (Carlsbad, CA). Purified human cord blood AFP (purity, >95%; SDS-PAGE) and purified human albumin (purity, >97%; SDS-PAGE) were obtained from Calbiochem (San Diego, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Recombinant human GM-CSF and IL-4 were purchased from PeproTech (Rocky Hill, NJ).

**Monocyte purification and generation of monocyte-derived DCs**

Mononuclear cells were isolated from peripheral blood by centrifugation on Ficoll-Hypaque (Amersham Pharmacia, Uppsala, Sweden). Mononuclear cells from healthy individuals were incubated in 96-well plates in RPMI 1640 for 30 min, and nonadherent cells were removed by gentle wash. DCs were generated as described previously (18). Briefly, adherent cells were cultured in DC medium (RPMI 1640 supplemented with 10% FCS) containing GM-CSF (500 IU/ml) and IL-4 (250 IU/ml) and in the presence or absence of AFP or human serum albumin (HSA). On days 3 and 5, the cells were fed with the DC medium and the above cytokines. Where indicated, AFP or HSA was added on day 5 to study the effect of AFP on different stages of DC differentiation. DC maturation was induced by the addition of LPS (500 ng/ml; Sigma-Aldrich) to the culture on day 7 for 24 h.

**Analysis of DC surface markers and apoptosis**

DCs were stained with FITC- or PE-labeled mAbs (anti-human CD1a, CD11c, CD14, CD19, CD20, CD40, CD80, CD83, HLA-DR, or relevant isotype controls; BD Pharmingen, San Diego, CA) according to the manufacturer’s instructions. Cells were gated according to their size (forward light scatter) and granularity (side light scatter) using a FACScan flow cytometer (BD Immunocytometry Systems, San Diego, CA). The DC surface marker expression was analyzed using the CellQuest program (BD Immunocytometry Systems). DC apoptosis was detected using Annexin V FITC, with dead cells identified by propidium iodide (PI) staining (BD Pharmingen).

**Mixed leukocyte reaction**

To avoid DC maturation, DCs were harvested following gamma irradiation. Gamma-irradiated allostimulatory DCs were incubated in round-bottom microtiter plates with 10^5 allogeneic T cells. Triplicate cultures were maintained for 5 days at 37°C in a 5% CO₂ humidified atmosphere. T cell proliferation was measured by pulsing cells with 1 μCi of methylthymidine (Amersham Pharmacia) for 18 h.

**Intracellular cytokine assay**

PBMCs or DCs were cultured in a medium containing LPS (500 ng/ml) and brefeldin A for 3 h. The cells were stained with anti-HLA-DR Ab, permeabilized, fixed, and stained with anti-human IL-12, IL-10, TNF-α, or isotype control Abs. The expression of intracellular cytokines was analyzed using flow cytometry.

**FIGURE 1.** DCs treated with AFP express low levels of CD86 molecules. Monocytes were cultured with GM-CSF and IL-4, in the presence or absence of different concentrations (25,000 ng/ml in a and c; 12,500, 5,000, or 2,500 ng/ml in b) of AFP or 25,000 ng/ml HSA. Cells were harvested on day 7 of culture, stained with different mAbs, and analyzed using flow cytometry. Cells were stained with PE-labeled mAbs. A PE-labeled, isotype-matched control Ig was used (a–c). Cells were also stained with FITC-labeled anti-HLA-DR and PE-labeled anti-CD14, CD16, CD19, and CD20 (a). Numbers represent the percentage of cells expressing CD83, CD86, CD40, HLA-DR, and CD11a. Sideward light scatter (SSC) and forward light scatter (FSC) characteristics are shown (a and b). Results were similar in four different independent experiments. On day 7, cells were treated with LPS to induce DC maturation, and the expression of surface molecules was analyzed on day 8 (c). Results were similar in three different independent experiments.
ELISA

Levels of biologically active IL-12 p70 and nonactive IL-12 p40 were measured using ELISA kit obtained from R&D Systems (Minneapolis, MN). ELISAs were performed in duplicate according to the manufacturer’s instructions.

Results

**AFP impairs DC function in vitro**

In this study, we have examined the effects of AFP on the function of monocyte-derived DCs generated from healthy individuals. AFP was added at concentration similar to that of AFP level reported in the sera of patients with HCC. As assessed by flow cytometry, monocyte-derived DCs cultured for 7 days in the presence of GM-CSF and IL-4 developed into DCs characterized by the acquisition of CD1a and CD11c and loss of CD14 molecules. They expressed high levels of HLA-DR and CD1a molecules but did not express CD14, CD16, CD19, or CD20 (Fig. 1a). Addition of AFP (as low as 2500 ng/ml) on day 0 of culture induced phenotypical alteration of DCs (Fig. 1b). However, this was not demonstrated with the addition of HSA. CD86 expression was substantially down-regulated, and a slight reduction of CD40 median fluorescence was detected. The expressions of CD1a, HLA-DR, and CD83 did not alter (Fig. 1a). Similar results were obtained when DCs were cultured in a medium containing 10% human serum (data not shown). When AFP was added on day 5 or 6 instead of day 0, a less pronounced decrease in CD86 expression was observed (data not shown). Addition of LPS on day 7 resulted in up-regulation of CD40, CD83, and CD86 molecules 24 h later (Fig. 1c). However the expression levels of the surface molecules on the AFP-treated mature DCs were still significantly lower than that of the nontreated mature DCs (Fig. 1c).

**AFP-treated DCs are poor stimulators of allogeneic T cell response in vitro**

AFP-treated DCs, but not HSA-treated or nontreated DCs, were shown to have low allostimulatory capacity. Graded numbers of viable DCs were cocultured with allogeneic T cells for 5 days in an MLR assay. The allostimulatory function of AFP-treated immature DCs was significantly reduced in a dose-dependent manner (Fig. 2, a and b). HSA did not inhibit allostimulatory function of DCs. A significant inhibitory effect of AFP (25,000 ng/ml) was also detected when AFP was added on day 5 (data not shown).

AFP-treated immature DCs were stimulated with LPS for 24 h to induce DC maturation. The allostimulatory ability of AFP-treated mature DCs (10,000 cells/well) was analyzed. AFP-treated mature DCs had significantly lower T cell-stimulatory capacity than that of nontreated mature DCs (Fig. 2c).

**AFP induces apoptosis of DCs in vitro**

The actual DC and PBMC recovery was determined after 7-day culture in the presence or absence of AFP. The cell recovery in the AFP-treated DCs, but not AFP-treated PBMCs, was significantly reduced. In the DC cultures, the number of cells recovered was 113,000 ± 10,000 for the AFP-treated group, and 236,000 ± 10,000 for the nontreated group. In PBMC cultures, the number of cells recovered was 143,000 ± 12,000 for the AFP-treated group, and 123,000 ± 4,000 for the nontreated group. The reduction of cell viability in the DC cultures after exposure to AFP may be due to induction of apoptosis. To investigate this possibility, cells were treated with AFP on day 0 of culture and analyzed on day 7 for the presence of apoptotic cells. AFP induced a significant increase in the number of apoptotic DCs as assessed by Annexin VFITC/PI staining (Fig. 3a). To test whether AFP exerts apoptotic effects on...
lymphocytes, PBMCs were treated with AFP for 7 days and annexin V binding was assessed on CD3^+ cells. AFP did not induce apoptosis of CD3^+ cells (Fig. 3a).

AFP inhibitory effects on DCs could be due to the induction of DC apoptosis or the suppression of DC function or both. We characterized the phenotype (Fig. 3b) and the function (c) of nonapoptotic DCs. The cells were gated on annexin V-negative, PI-negative (nonapoptotic), and the expression levels of CD86 molecule were analyzed. The nonapoptotic DCs exposed to AFP expressed slightly lower levels of CD86 than that on nonapoptotic, nontreated DCs (Fig. 3b). To test the functional ability of nonapoptotic DCs, we first determined the percentage of apoptotic cells in the cultures (annexin V-positive, PI-negative cells). The ability of nonapoptotic cells to stimulate T cell proliferation was determined in an MLR assay. The number of nonapoptotic cells in unsorted cells was estimated, and AFP-treated or nontreated nonapoptotic DCs, i.e., annexin V-negative, PI-negative cells, were added (20,000 cells/well) to allogeneic T lymphocytes. After 5 days, T cell proliferation was assessed by the addition of thymidine for 18 h (c). The data shown are average counts per minute of three replicate determinations ± SD. The data are representative of three similar experiments.

FIGURE 3. AFP induces apoptosis of monocyte-derived DCs but not in CD3^+ cells. Monocytes were cultured in the presence of GM-CSF and IL-4, and AFP or HSA (25,000 ng/ml) was added on day 0 of culture. PBMC were also cultured in the presence of AFP or HSA (25,000 ng/ml) for 7 days. A two- or three-color staining with annexin V and PI with (a) or without (a) anti-CD86 Ab was conducted on day 7 of DC culture. A three-color staining was performed on PBMC culture (annexin V, PI, and anti-CD3 Ab). Numbers represent the percentage of cells in each quadrant. The cells in PBMC culture were gated on CD3^+ cells, and the expression of annexin V-positive, PI-negative cells (apoptotic cells) was analyzed (a). The cells in DC culture were gated on nonapoptotic cells (annexin V-negative, PI-negative), and the expression levels of CD86 molecules were analyzed (b). The counted cells for the AFP-treated group were 340,000 cells, and for nontreated and HSA-treated group was 10,000 cells. AFP-treated or nontreated nonapoptotic DCs, i.e., annexin V-negative, PI-negative cells, were added (20,000 cells/well) to allogeneic T lymphocytes. After 5 days, T cell proliferation was assessed by the addition of thymidine for 18 h (c). The data shown are average counts per minute of three replicate determinations ± SD. The data are representative of three similar experiments.

DCs were stimulated with LPS for 3 h, and the production of intracellular TNF-α, IL-12, and IL-10 was analyzed. DCs that were differentiated in the presence of AFP had an impaired ability to produce IL-12 and TNF-α. The lowest level of AFP used in vitro with inhibitory effects on IL-12 production was 12,500 ng/ml. HSA treatment did not significantly reduce the ability of DCs to
produce TNF-α and IL-12. When AFP was added on day 5 instead of day 0 of culture, a significant decrease in IL-12 production occurred. However, a greater decrease was seen when AFP was added on day 0 (Fig. 4). The serum AFP levels in patients with HCC (n = 16) are shown in Fig. 5. The lowest amount of AFP used in vitro to induce CD86 down-regulation on DC was 2,500 ng/ml and for inhibition of IL-12 production was 12,500 ng/ml. There was an undetectable level of IL-10-producing cells in all three experimental groups (data not shown).

The level of IL-12 (p70) and IL-12 (p40) in supernatant of AFP-treated DCs (1 × 10^5/well) was measured using ELISA after 48-h stimulation with LPS. AFP-treated DCs produced lower levels of IL-12 (p40) and bioactive IL-12 (p70) than that of nontreated DCs. No IL-12 (p40) or IL-12 (p70) was detected in the supernatant of AFP-treated DCs. The levels of IL-12 (p40) and IL-12 (p70), produced by nontreated DCs, were 2200 ± 230 and 508 ± 127 pg/ml, respectively.

**APCs of patients with HCC produce low levels of TNF-α ex vivo**

Flow cytometry was used to examine TNF-α secretion profiles of PBMCs in patients with HCC (n = 16) and healthy individuals (n = 7), in response to stimulation with LPS. The cells were gated on HLA-DR-positive or CD14-positive cells, and the expression of intracellular TNF-α was determined. In both healthy individuals and HCC patients, 70–98% of TNF-α-producing cells expressed high levels of HLA-DR molecule and 71–93% expressed CD14 molecule. The percentage of TNF-α-producing cells was lower in HCC patients with high levels of serum AFP than that of the healthy group (Fig. 6). To examine whether the reduction of TNF-α production could be attributed to the loss of HLA-DR-positive cells, the percentage and mean fluorescence intensity of cells expressing HLA-DR molecules were analyzed. No significant loss of HLA-DR-positive cells was observed (Fig. 6, b and c). When the ability of HLA-DR-positive cells to produce IL-10 was analyzed, there was no significant difference between patients with HCC and healthy individuals (data not shown).

**Discussion**

Inadequate presentation of tumor Ags by the host professional APCs is one potential mechanism by which tumors evade the
entiation in vivo. We used a concentration of AFP (2,500–25,000 ng/ml) in PBMCs of HCC patients with high levels of serum AFP produced lower TNF-α ex vivo. PBMCs were stimulated with LPS for 3 h, harvested, and stained with FITC-labeled anti-HLA-DR mAb for surface molecules and PE-labeled TNF-α for intracellular cytokine. Cells were analyzed using flow cytometry, and the percentages of HLA-DR "TNF-α" cells were determined. The closed dots (■) represent results obtained from patients with HCC (n = 16), and open dots (○) represent results obtained from healthy controls (n = 7), with the y-axis representing percentage of HLA-DR "TNF-α" cells and the x-axis representing the levels of serum AFP (a). The percentage (b) and mean fluorescent intensity (c) of cells expressing HLA-DR molecules in PBMC of patients with HCC and healthy individuals are shown.

In conclusion, this study demonstrates for the first time that AFP severely impairs the function of DCs and induces their apoptosis. In addition, the ability of APCs, of patients with HCC and high levels of serum AFP, to produce proinflammatory cytokines is reduced. This provides new insights into understanding the mechanisms underlying the suppression of immune recognition of tumors in patients with HCC.

References