Retinoids Act as Multistep Modulators of the Major Histocompatibility Class I Presentation Pathway and Sensitize Neuroblastomas to Cytotoxic Lymphocytes

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ABSTRACT

The current therapeutic modalities achieve low response rates in human neuroblastoma, a frequent extracranial malignancy of the early childhood. We have assessed the effect of retinoids, used presently for the treatment of neuroblastoma, on the discrete steps of the MHC class I processing machinery and susceptibility of neuroblastoma cells to CTL-mediated killing. We demonstrate that retinoic acid derivatives induce the expression of proteolytic and regulatory subunits of the immunoproteasome, increase the half-life of MHC class I complexes, and enhance the sensitivity of neuroblastoma cells to both MHC class I-restricted peptide-specific and HLA nonrestricted lysis by CTLs. Importantly, effects of retinoids on the MHC class I pathway appear to be independent of IFN-γ and/or TNF-α as intermediate messengers. To our knowledge, this is the first demonstration of inflammation-unrelated biological molecules that induce systemic modulation of antigen presentation in nonprofessional antigen presenting cells. Our findings suggest that the application of retinoids and T cell-based immunotherapy may be an effective combination for the treatment of neuroblastoma.

INTRODUCTION

Neuroblastoma is a frequent extracranial malignancy of the early childhood. Current standard approaches to the treatment of neuroblastoma include surgery, chemotherapy, and radiation, whereas immune-based therapies are limited to immunotherapy directed at minimal residual disease (1, 2). Treatment modalities of neuroblastoma based on tumor-specific cytotoxic T-lymphocytes have not yet been developed.

Several lines of evidence support the critical importance of the CD8+ subset of T cells in the clearance of tumors or prevention of their development (3, 4). Mature cytotoxic T-cells (CTLs) are specific for peptides displayed on MHC class I molecules. The MHC class I presentation pathway involves proteolytic generation of antigenic peptides followed by peptide transport into the ER1 where MHC complex-peptide association takes place (reviewed in Refs. 5, 6). The main enzymatic complex that degrades ubiquitin-tagged proteins is the 26S proteasome (7). The proteolytically active sites are present in adjacent pairs of identical β-subunits (β1, β2, and β5), and represent trypsin-like, chymotrypsin-like and postglutamyl peptidyl hydrolytic activities. After stimulation of the antigen-presenting cell with IFN-γ, β1, β2, and β5 subunits are substituted by LMP2, MECL1, and LMP7, respectively. This leads to alterations in the cleavage site preferences of the proteasome, and an increase in the production of peptides with basic and hydrophobic COOH termini (8), better uptake of peptides by TAPs, and better binding to the MHC class I molecule. The latter results in an increased level of expression and higher stability of MHC class I complexes at the surface of target cells that promotes more efficient T cell-mediated recognition.

Physiological signals able to increase and stabilize the pool of surface MHC class I molecules in tumor cells are of special interest. Data available on this subject mainly concern effects obtained with proinflammatory cytokines, such as IFN-γ (reviewed in Ref. 9) and TNF-α (10). Vitamin A derivatives (retinoids) were reported to increase the total pool of surface HLA class I complexes in mammalian cells; however, the molecular basis underlying this phenomenon was not studied in detail (11–13). Retinoids are known as potent modifiers of proliferation and differentiation in different cell types, including neuroblastoma (14, 15). After in vitro studies that demonstrated retinoid-induced growth arrest and differentiation of neuroblastoma, several clinical trials revealed the ability of retinoids to increase survival in neuroblastoma patients. However, preclinical studies in neuroblastoma indicate that all-trans-RA or 13-cis-RA can antagonize cytotoxic chemotherapy and radiation. Therefore, the use of RA derivatives in neuroblastoma is limited to the maintenance therapy after completion of these treatment modalities (reviewed in Ref. 16).

In view of the fact that the currently available therapeutic modalities induce low response rates in human neuroblastomas, we have evaluated the potential usefulness of retinoids in combination with T cell-based therapy of neuroblastoma. We have assessed the effect of retinoids on the discrete steps of the MHC class I processing machinery and analyzed the outcome of RA treatment on the susceptibility of neuroblastoma cells to the effector mechanisms of CTLs and NK cells.

MATERIALS AND METHODS

Cell Lines

Tumor Cell Lines. The neuroblastoma cell lines CHP-212, SK-N-DZ, SK-N-BE (2), SH-SY5Y, MC-IXC, SK-N-AS, SK-N-SH, and IMR-32 were purchased from American Type Culture Collection. The neuroblastoma cell lines Ln1 and Lan5 were kindly provided by Dr. Marie Henriksen-Arnesen (Karolinska Institutet, Stockholm, Sweden). The FL-2 cell line, a subclone of SH-SY5Y, was provided by Dr. Marianne Ifversen (Rigshospitalet, Copenhagen, Denmark).

The cell lines were maintained in IMDM supplemented with 10% heat-inactivated FCS (Life Technologies, Inc., Grand Island, NY), 100 IU/ml penicillin, and 100 μg/ml streptomycin (complete medium).

Treatment with RA derivatives was performed at a final concentration of 10 μM in complete medium. Cells kept in complete medium containing the corresponding amount of DMSO are thereafter referred to as “control.”

Effectors. The generation and characterization of the CD8+ HLA A11-restricted CTL clone BK289, specific for the EBV nuclear antigen-4-derived peptide IVT, were described previously (17).

Purification of NK cells and generation of polyclonal NK cell cultures was performed from peripheral blood lymphocytes of healthy donors by density gradient centrifugation on Ficoll-Hypaque with subsequent elimination of cells.

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The abbreviations used are: ER, endoplasmic reticulum; TAP, transporter associated with antigen presentation; TNF, tumor necrosis factor; RA, retinoic acid; NK, natural killer; IVT, IVTDFSVIK; FACS, fluorescence-activated cell sorter; ABC, avidin-biotin complex method; BFA, Brefeldin A; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; Fas-L, Fas ligand.
adherent to plastic. The remaining cell population was incubated on ice with the mixture of monoclonal antibodies including anti-CD3 (JT3A), anti-CD4 (HP2.6), and anti-HLA-DR (D1.12) followed by goat antimouse IgG-coated Miltenyi Microbeads (Miltenyi Biotech GmbH). After immunomagnetic depletion, FACS analysis of the resulting cell pool was performed demonstrating that 90–95% of these cells were CD3⁺, CD4⁺, and HLA-DR⁻ (data not shown). Cells were cultured with irradiated allogeneic feeders in the presence of 200 units/ml of recombinant interleukin 2 and 1.5 ng/ml phytohemagglutinin (Life Technologies Inc., Paisley, Scotland).

**Antibodies and Chemicals.** RA derivatives 9-cis-RA and all-trans-RA were purchased from Sigma (St. Louis, MO) and Ro 13-6307 (Ro13) was obtained from Hoffmann-La Roche (Basel, Switzerland), dissolved in DMSO as 100 mM stocks, and stored at −70°C in small aliquots.

The HLA ABC-specific antibody (clone W6/32) conjugated with R-phycocerythrin (RPE), RPE-conjugated mouse IgG2a isotype antibodies, and FITC-conjugated rabbit antimouse F(ab')2 fragments were obtained from DAKO-PATTS AB (Alvsjö, Sweden). Hybridomas producing the HLA A11-specific antibody A11.1M (clone HB-164) and HLA-A2-specific antibody MA2.1 (clone HB-54) were from the American Type Culture Collection. Total mouse serum was prepared by the animal facility at the Microbiology and Tumorbiology Center, Karolinska Institutet.

Antibodies specific to LMP2, LMP7, MECL-1, and PA28γ subunits were purchased from Affinity Research Products Ltd. (Mamhead Castle, Exeter, United Kingdom). Rabbit polyclonal serum specific to human class I heavy chain was a kind gift from Dr. Hidde Ploegh (Department of Pathology, Harvard Medical School, Boston, MA). Human recombinant TNF-α was obtained from Cetus Corporation (Emeryville, CA) and IFN-γ (Imukin) from Boehringer Ingelheim International GmbH (Ingelheim, Germany). BFA and actin-specific antibodies were purchased from Sigma (Sigma Chemical Co.). IFN-γ blocking antibodies were from Nordic Biosite (Täby, Sweden). Soluble recombinant TNF-receptor 2 (Enbrel) was a kind gift of Prof. Lars Klareskog (Karolinska Institutet, Center for Molecular Medicine, Stockholm, Sweden).

**Western Blot Analysis.** All of the procedures were performed using Multiphor II Electrophoresis System and ExCelGel SDS homogeneous precast gels (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Neuroblastoma cells cultured in complete medium containing DMSO (1 μl in 10 ml) or in the presence of 9-cis-RA (10 μM) for 96 h at 37°C were pelleted down and lysed in electrophoresis sample buffer. Aliquots of total cell lysates corresponding to 10⁶ cells were separated by SDS-PAGE followed by transfer onto polyvinylidene difluoride membrane (Millipore AB, Sundbyberg, Sweden). Membranes were blocked in PBS containing 5% milk and 0.1% Tween 20, and probed with the indicated specific antibody at the dilution recommended by the manufacturer. The following secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech AB) were used: antirabbit for LMP2, LMP7, MECL-1, and PA28γ. The reaction was visualized by enhanced chemiluminescence according to the manufacturer’s protocol (Amersham Pharmacia Biotech AB).

**Cytotoxicity Assays.** Standard 4 h ⁵¹Cr release assays were performed as described previously (18). Briefly, HLA-A11-positive neuroblastoma cell lines were preincubated with the IVT-peptide at a range of peptide concentrations described previously (18). Briefly, HLA-A11-positive neuroblastoma cell lines were preincubated with the IVT-peptide at a range of peptide concentrations described previously (18). Briefly, HLA-A11-positive neuroblastoma cell lines were preincubated with the IVT-peptide at a range of peptide concentrations described previously (18).

**RESULTS**

RA Derivatives Increase the Levels of HLA ABC Complexes at the Cell Surface of Neuroblastoma Cells. The density of specific ligand at the surface of target cells is one of the main parameters determining the degree of CTL activation. Therefore, the ability to induce MHC class I complexes at the cell surface was chosen to assess the immunomodulating capacity of RA on different cell lines used in our study.

We found that ~50% of neuroblastomas (thereafter referred to as...
“responders”) responded to 9-cis-RA treatment by up-regulating (more than 1.5 fold) the HLA class I at the cell surface (Table 1; Fig. 1). In agreement with this observation, the total pool of MHC class I heavy chain in cell lysates of RA-treated cells was also increased (Fig. 3). The induction of class I molecules in neuroblastomas was not specific for one RA-derivative only and could also be seen with all-trans-RA and Ro 13–6307 (Fig. 2).

A proportion of neuroblastomas (thereafter referred to as “nonresponders”) did not change the levels of surface MHC class I on RA treatment (Table 1) and failed to induce the total pool of class I heavy chain (Fig. 3). This nonresponsiveness was affected by neither the increased concentrations nor the repeated treatment with different retinoids for a period of 6–8 days (data not shown). This phenomenon could not be explained by the general resistance of these cells to RA treatment, because morphological changes compatible with RA-induced cell differentiation were observed upon culturing of nonresponders in the presence of different RA derivatives, such as 9-cis-RA, all-trans-RA, and Ro 13–6307 (data not shown).

The antibody specific for HLA ABC (clone W6/32) used in our experiments recognizes classical as well as nonclassical HLA molecules, such as HLA-E and HLA-G. Therefore, we checked whether the up-regulation of individual alleles, such as HLA-A2 or HLA-A11, known to be important restriction elements in CTL-mediated tumor recognition (19), contributed to the increase of class I complexes on RA treatment. Indeed, both HLA-A11 (Fig. 1C) and HLA-A2 (Fig. 1D) were efficiently induced by 9-cis-RA in SK-N-SH and CHP-212 cell lines, respectively.

**RA Increases the Stability of MHC Class I Complexes in Neuroblastoma Cells.** The increased amounts of heavy chain on RA treatment may result from the induction of transcription and/or transliteration of this molecule, or may reflect the increased half-life of MHC class I complexes. The latter is favorable for recognition of tumors by CTLs (20–22). We monitored the effect of RA on the stability of class I complexes in total cell lysates (Fig. 4, A and B) and at the cell surface of neuroblastoma cells (Fig. 4C). We found that the half-life of class I molecules in lysates of 35S-labeled SK-N-SH cells increases from 8–10 h to 18–20 h on RA treatment. In agreement with this observation, the MHC class I complexes were more stable at the cell surface of RA-treated as compared with control SK-N-SH cells (Fig. 4C). Not more than 6–8% of the initial amount of MHC I complexes were lost from the cell surface during 6 h after BFA treatment in 9-cis-RA-treated samples, whereas HLA ABC levels in control cells were already reduced by 20%. Additional comparative analysis of the surface complex stability appeared to be thwarted due to high toxicity of BFA for neuroblastoma cells.

**RA Induces Expression of the Immunoproteasomal Subunits in Neuroblastoma Cells.** Presentation of most MHC class I-restricted peptide epitopes is proteasome-mediated and TAP-dependent (23, 24). Therefore, we investigated the effects of RA on the expression of the immunoproteasomal subunits in neuroblastoma cells. The expres-

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**Table 1 RA derivatives increase the expression of MHC class I complexes at the surface of neuroblastoma cells**

Neuroblastoma cell lines were treated with 9-cis-RA for 72 h, and surface expression of MHC class I complexes was monitored by FACS using W6/32 antibody specific to HLA ABC. The MFI obtained with the isotype control (MFI isotype) and W6/32 (MFI HLA ABC) antibody are shown for each cell line. The ratio between the mean fluorescence intensity obtained in the control and RA-treated samples was calculated and expressed as fold change in MHC class I expression. Data of one representative of three to six performed experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MHC change (fold)</th>
<th>Responder (+/−)</th>
<th>Control</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-BE(2)</td>
<td>1.1</td>
<td>−</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>Lan5</td>
<td>0.9</td>
<td>−</td>
<td>5</td>
<td>925</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>2.0</td>
<td>+</td>
<td>6</td>
<td>81</td>
</tr>
<tr>
<td>MC-IXC</td>
<td>6.5</td>
<td>+</td>
<td>4</td>
<td>320</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>1.7</td>
<td>+</td>
<td>3</td>
<td>1267</td>
</tr>
<tr>
<td>FL2</td>
<td>1.1</td>
<td>−</td>
<td>4</td>
<td>913</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>6.4</td>
<td>+</td>
<td>4</td>
<td>189</td>
</tr>
<tr>
<td>SK-N-DZ</td>
<td>1.1</td>
<td>−</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>IMR32</td>
<td>1.1</td>
<td>−</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>Lan1</td>
<td>0.8</td>
<td>−</td>
<td>9</td>
<td>339</td>
</tr>
<tr>
<td>CHP-212</td>
<td>3.1</td>
<td>+</td>
<td>6</td>
<td>340</td>
</tr>
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</table>

a MFI, mean fluorescence intensity.
A pulse-chase experiment shown in A intensity of the specific band at the indicated time point/intensity of the specific band at materials and Methods. B, specificity of the proteasome. Changes in the proteolytic activity can after RA treatment (Fig. 5), suggesting alterations in fine cleavage specificity of the proteasome. Changes in the proteolytic activity can also be induced on association of the constitutive proteasomal core with the nonproteolytic regulatory complex PA28α (25). We observed a coordinated induction of the PA28α regulator, suggesting that both proteolytic and regulatory functions of the proteasome in neuroblasto- 

mias identified by us as responders, undergo changes on exposure to retinoids. In contrast, the expression of inducible catalytic and regulatory proteasomal subunits was not modified in nonresponders, such as SK-N-BE and SK-N-DZ cell lines (Fig. 5).

The Effect of RA on MHC Class I Antigen Processing in Neuroblastomas Is Not Dependent on IFN-γ and TNF-α. The proinflammatory cytokines IFN-γ and TNF-α up-regulate the expression of MHC class I complexes, immunoproteasomal subunits, and TAP1/TAP2 in a variety of cell types. The effects of RA exerted on the antigen processing machinery in neuroblastoma cells closely resembled that seen on treatment with IFN-γ and TNF-α. To identify whether or not the RA-induced changes in the MHC class I pathway of neuroblastomas reflect the induction of endogenously produced proinflammatory cytokines, we performed RA treatment of neuroblastomas in the presence of IFN-γ or TNF-α-blocking agents (Fig. 6). Treatment with 5IU/ml of IFN-γ caused a 10-fold induction of HLA ABC at the surface of SK-N-SH cells, which was completely abolished in the presence of an IFN-γ-blocking antibody (Fig. 6A). The same amount of the antibody had no effect on the significantly weaker MHC class I induction achieved by RA treatment (Fig. 6B). Furthermore, we could not detect mRNA of IFN-γ by reverse transcription-PCR neither in untreated nor RA-treated SK-N-SH cells (data not shown). Like IFN-γ, TNF-α up-regulated surface HLA ABC complexes on SK-N-SH cells to the levels achieved on RA treatment. TNF-α-induced up-regulation of MHC class I was completely prevented by the recombinant soluble TNF-R2 (Fig. 6C), whereas the same reagent had no effect on 9-cis-RA-treated tumor cells (Fig. 6D). Similar results were achieved using the IFN-γ and TNF-α blocking agents on RA-treated MC-IXC cells (data not shown).

Neuroblastoma Cells Exposed to RA Are Lysed More Efficiently by Peptide-Specific MHC Class I-Restricted CD8+ CTLs. We next investigated the effect of retinoids on the recognition of neuroblastoma by peptide-specific MHC class I-restricted CTLs. Both SK-N-SH and MC-IXC cell lines are HLA-A11 positive, therefore, the tumor cells were pulsed with the IVT peptide at different concentrations and coincubated with BK 289 CTL (17) at an E:T ratio of 1:1 in a standard 4 h 51Cr release assay. As shown in Fig. 7, A and B, neuroblastoma cells treated with RA were more efficiently lysed by CTLs at a range of peptide concentrations. This effect was peptide-specific because no recognition of either DMSO- or RA-treated neuroblastomas was observed by the same effectors in the absence of HLA-A11-restricted epitope (Fig. 7G). Noteworthy, we did not observe any significant difference in the CTL-mediated killing on RA treatment of two HLA-A11-positive nonresponder cell lines SK-N-BE and SK-N-FI (Fig. 8). A significant (~30-fold) up-regulation of HLA-A11 was induced by RA in SK-N-SH cells (Fig. 7C) used for

Fig. 3. Effect of RA on the expression of the total pool of heavy chain in neuroblastoma cells. The expression of the MHC class I heavy chain in total cell lysates of neuroblastoma cell lines was monitored by Western blot before and after RA-treatment. Expression of actin was used as a control of loading.

Fig. 4. RA increases the stability of MHC class I complexes in neuroblastoma cells. A, MHC class I complexes in the total cell lysate of metabolically labeled SK-N-SH cells either untreated (control) or treated with 9-cis-RA were monitored in pulse-chase experiments using immunoprecipitation with subsequent autoradiography as described in “Materials and Methods.” B, percentage of MHC class I expression at each time point of the pulse-chase experiment shown in A was calculated from densitometric analysis as follows: intensity of the specific band at the indicated time point/intensity of the specific band at time zero ×100. C, stability of MHC class I complexes at the cell surface of SK-N-SH cell line. The amount of class I molecules at the cell surface of RA-treated (light bars) and control cells (dark bars) was monitored after 2, 4, and 6 h after exposure to BFA. Mean fluorescence intensity for each sample was calculated as a difference between the values obtained with W6/32 and isotype control antibody. The resulting intensity of fluorescence at each time point is shown as a percentage decrease relative to the intensity of fluorescence in cells before BFA-treatment (indicated as % MHC decrease). One representative experiment of three performed. The following designations are used in the figure: RA (cells pretreated with 10 μM of RA for 72 h), control (DMSO-treated cells).

Table: RA effects on the expression of heavy chain and actin

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>RA</th>
<th>Heavy chain</th>
<th>Actin</th>
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<tbody>
<tr>
<td>MC-IXC</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CHP-212</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>SK-N-BE</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SK-N-DZ</td>
<td>-</td>
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MC-IXC, CHP-212, SK-N-SH, SK-N-BE, SK-N-DZ

4 J. Levitskaya, unpublished observations.
the CTL assay (Fig. 7A). However, MC-IXC cell line did not significantly up-regulate HLA-A11 in response to RA treatment, probably due to the relatively high steady-state expression level of this molecule at the cell surface (Fig. 7D). We surmised that in this situation the modulation of the expression of adhesion molecules may affect the outcome of CTL-mediated cytotoxicity. In fact, the adhesion molecule ICAM-1 was significantly (up to 10-fold) up-regulated in both neuroblastoma cell lines on RA-treatment (Fig. 7, E and F), thus correlating with the increased lysis by CTLs.

**RA Increases Susceptibility of Neuroblastoma Cells to the By-stander Cytotoxicity of CTLs.** Activated CTLs express a number of effector molecules capable of inducing apoptosis in the target in a HLA-nonrestricted manner. These include: Fas-L, TRAIL, IFN-γ and TNF-α (26-28). The CTL clone BK 289 used in our study possesses the phenotype of activated memory T cells, and expresses Fas-L and TRAIL (data not shown). We investigated whether RA can interfere with the sensitivity of tumors to death receptor-mediated apoptosis induced by CTLs in a bystander fashion. Neuroblastoma cells, either untreated or treated with 9-cis-RA, were cocultured with BK 289, and 51Cr release was measured after 16 h. Only relatively weak lysis of SK-N-SH was detected (at maximum 20% at 5:1 E:T ratio), whereas MC-IXC cells were not recognized. Pretreatment of tumor cells with 9-cis-RA resulted in a 3-4-fold more efficient killing by CTLs (Fig. 9).

**RA Treatment Facilitates Tumor Lysis by NK Cells Despite the Up-Regulation of MHC Class I at the Cell Surface.** High density of HLA class I at the surface of neuroblastoma cells may serve as an inhibitory signal preventing activation of NK cells. For that reason we examined the sensitivity of RA-treated targets to NK-mediated recognition. Two types of effectors, NK cell line Nishi (Fig. 10A) and polyclonal NK cultures obtained from the blood of healthy donors (Fig. 10B), were used against either untreated or RA-treated SK-N-SH cells as targets in a 4-h 51Cr release assay. Despite 5-fold up-regulation of HLA class I at the cell surface in RA-treated samples (data not shown), these cells were either equally sensitive to recognition by Nishi cells (Fig. 10A) or appeared to be more efficiently lysed by freshly isolated NK cultures at a range of E:T ratios (Fig. 10B).

**DISCUSSION**

Tumor-specific responses mediated by CD8+ CTLs provide an example of highly specific recognition of the unique peptide structures associated with HLA molecule at the surface of malignant cells followed by the rapid and directed release of cytolytic granules (26), induction of ligands for death receptors, such as Fas-ligand and TRAIL (27), and production of cytokines that either facilitate the CTL effector functions mentioned above, or serve as inducers of apoptosis in the tumor target (28, 29). Development of new methods for improving MHC class I-restricted antigen presentation in malignant cells represent one of the major tasks of tumor immunology. A few biological molecules are known to act as positive regulators of the MHC class I presentation machinery. These include IFN-γ and TNF-α (9, 10) mainly produced by the effector cells of the immune system at the sites of inflammation. Here we demonstrate that RA derivatives induce the expression of different components of the MHC class I presentation machinery in neuroblastoma cells (Figs. 3 and 5), increase the half-life of MHC class I complexes (Fig. 4), and enhance the sensitivity of neuroblastoma cells to lysis by CTLs, both in the MHC class I-restricted (Fig. 7) and HLA-nonrestricted fashion (Figs. 9 and 10). Importantly, the effects of retinoids on the MHC class I pathway in neuroblastomas did not involve IFN-γ and/or TNF-α as intermediate messengers (Fig. 6). To our knowledge, this is the first demonstration of inflammation unrelated, naturally produced biological molecules that induce systemic modulation of antigen presentation in nonprofessional antigen-presenting cells.

A number of reasons prompted us to investigate the effects of RA on the MHC class I processing and presentation in neuroblastomas. First, retinoids are currently in use as a treatment modality in neuroblastoma patients. Second, a systematic analysis of the antigen presentation in tumor cells after administration of retinoids was never performed before, thus limiting the possibility to assess the potential of combining RA treatment with T cell-based immunotherapy of neuroblastoma.

During the past decade several reports addressed the effect of RA on the immunogenicity of tumors of various origins but were usually limited to monitoring the expression of surface HLA and adhesion molecules. These studies generated controversial results. Several groups have demonstrated that the exposure to therapeutic doses of RA was able to significantly increase the expression of MHC class I and/or adhesion molecules, such as ICAM-1 and lymphocyte function
antigen-3 (LFA-3) in human cervical carcinoma (15, 30), melanoma, glioma, teratocarcinoma (11), neuroblastoma cell line SK-N-SH (12), human glioblastoma cell lines (13), and myelocytic leukemia cells (31). Others claimed that RA exhibited no effect on the expression of cell surface molecules, including HLA class I and class II antigens, ICAM-1 and -2, and LFA-3 (13, 32). Moreover, several studies have demonstrated down-regulation of HLA molecules on RA-induced differentiation of a human embryonic stem line (33).

The density of specific peptide:MHC complex at the surface of the target is one of the crucial parameters determining the efficacy of CTL activation. For that reason, surface expression of total HLA ABC in neuroblastomas was chosen by us as a parameter of responsiveness of the MHC class I presentation pathway to RA treatment (Table 1). The up-regulation of the total pool of MHC class I molecules in 5 of 11 neuroblastoma lines varied from 1.6- to 6.5-fold. Importantly, an increase of individual HLA alleles such as HLA-A2 and HLA-A11 was also observed (Fig. 1), additionally validating the results obtained with the W6/32 antibody cross-reacting with nonclassical HLA alleles. The W6/32 antibody used in our study recognizes class I heavy chains in association with β2m; therefore, our results contradict a previously published observation that differentiation of neuroblastomas with RA mainly induces MHC class I molecules not bound to the light chains (34). We failed to detect significant changes in the expression of surface MHC I complexes in 6 of 11 neuroblastoma lines even at the late time points of observation. The absence of MHC class I induction in these neuroblastoma lines could not be accounted for by a general nonresponsiveness to retinoids, because these cell lines exhibited morphological and biochemical changes compatible with growth arrest and/or apoptosis (data not shown). The mechanisms preventing the modulation of MHC class I in this group of neuroblastomas remain unclear.

Limited data are available on the nature of signals augmenting the half-life of MHC complexes in nonprofessional antigen-presenting cells. The ability of IFN-γ and TNF-α to the increase the stability of MHC class I complexes was demonstrated in both mouse and human cells (reviewed in Ref. 5). Here we report a >2-fold increase in the stability of MHC class I complexes after RA treatment that is independent of proinflammatory cytokines. The precise mechanisms of these changes remain to be investigated. It is tempting to speculate that formation of the "immunoproteasome" and induction of PA28α

![Fig. 7. RA treatment increases MHC class I-restricted peptide-specific lysis of neuroblastoma cells by CD8+ CTLs. The HLA-A11+ neuroblastoma cell lines SK-N-SH (A) and MC-IXC (B), either untreated (□) or treated with RA (●) for 96 h were prepulsed with the IVT peptide at the indicated concentrations and tested for sensitivity to lysis by the peptide-specific HLA-A11-restricted CD8+ CTL clone BK289 in a standard 4 h 51Cr release assay at 1:1 E:T ratio. Surface expression of HLA-A11 (C and D) and ICAM-1 (E and F) was determined by immunostaining with relevant antibodies (see "Materials and Methods") and FACS analysis. G, DMSO- or RA-treated SK-N-SH and MC-IXC cell lines either prepulsed with the 10^{-7} M of the IVT peptide or left unpulsed, were tested for sensitivity to lysis by the peptide-specific HLA-A11-restricted CD8+ CTL clone BK289 in a standard 4 h 51Cr release assay at 2:1 E:T ratio. Mean in each panel represent % specific lysis obtained from at least four experiments performed with each cell line; bars, ±SD.](image)
observed in RA-treated neuroblastomas (Fig. 5) may alter the peptide repertoire that is available for binding. Moreover, changes in the expression of MHC class I-associated chaperones and altered recycling of class I complexes may also contribute to this phenomenon. Notably, we failed to observe a detectable improvement in the expression of the TAP heterodimer after RA treatment (data not shown). This could be a result of remodeling of the ER influencing the expression of the ER-anchored proteins as observed in some models (36). Nevertheless, despite the absence of detectable changes in the expression of peptide transporters, some neuroblastomas exposed to retinoids had higher density and stability of the MHC class I complexes at the cell surface consistent with the sufficient supply of “optimal peptides” available for loading onto MHC (36).

The amount and quality of surface MHC class I complexes determine the outcome of recognition of tumor targets by different effectors cells of the immune system. The main T-cell subset dependent on these parameters is CD8+ CTLs. The data on the sensitivity of neuroblastomas to recognition and elimination by specific CTLs is limited. We found that RA treatment results in the enhanced lysis of neuroblastomas by peptide-specific MHC class I-restricted CTL clones at many peptide concentrations (Fig. 7, A and B). This correlated with the enhanced levels of the restriction element (HLA-A11) at the cell surface of RA-treated neuroblastoma (Fig. 7C) and/or elevated levels of the ICAM-1 molecule (Fig. 7, E and F). Although relatively weak (about 10–15%) up-regulation of HLA A11 was usually induced by RA in MC-IXC (Fig. 7D), this line became more sensitive to killing by CTLs (Fig. 7B), which may be explained by the significant induction of ICAM-1 (Fig. 7F). To our knowledge, this is the first demonstration of the effect of retinoids on the CD8+ CTL lysis of neuroblastomas, suggesting that treatment with retinoids and CTL-based immunotherapy may have a synergistic effect.

In contrast to CTLs, NK cells can be inhibited, rather than activated by high levels of MHC class I at the surface of tumor targets (37). It was also reported that all-trans RA decreases the susceptibility of a gastric cancer cell line, hepatic cancer, and promyelocytic leukemia cell lines to lymphokine-activated killer cytotoxicity (32). However, this was not the case for RA-treated neuroblastomas, because freshly isolated polyclonal NK cultures recognized RA-treated neuroblastomas more efficiently as compared with untreated cells (Fig. 10B). Several factors could contribute to this phenomenon; first, the elevated levels of adhesion molecules induced by RA (data not shown). Second, the RA-inducible molecule MICA that serves as a ligand for activating NKG2D receptors of human natural killer cells (reviewed in Ref. 38) has been known to override the inhibiting signal induced by high levels of MHC class I. MICA is expressed by neuroblastoma cell lines used in our study (data not shown), which may provide another explanation for the enhanced sensitivity of neuroblastoma cells to NK-mediated lysis. This assumption is in accordance with the previously published finding that RA-induced increase in the ICAM-1 levels were only partly responsible for the increase in susceptibility of tumor cells to LAK cells (39).

Characterization of the immune phenotype and cytotoxic activity of neuroblastoma-associated tumor-infiltrating lymphocytes has shown the presence of CD8+ and CD4+ T cells. Both T-cell populations were polyclonal, and cytokine production by CD4+ clones was of the T-help 1 profile and similar to that observed for CD8+ CTLs (40). The contact of tumor cells with tumor-infiltrating lymphocytes may result in the induction of apoptosis in an MHC class I-independent fashion via death receptors, such as FAS, TRAIL, and/or TNF receptors I and II. The MHC class-I nonrestricted “bystander” recognition of neuroblastoma cells by T cells was induced (Fig. 9B) or additionally up-regulated (Fig. 9A) by pretreatment of targets with 9-cis-RRA. Moreover, RA-treated neuroblastomas exhibited higher sensitivity to apoptosis induced by either soluble recombinant TRAIL or by the FAS-agonistic antibody CH11 (41) that was additionally enhanced when these molecules were applied in combination with TNF-α and IFN-γ (data not shown).

Collectively, our data suggest that treatment of neuroblastoma cells with RA derivatives facilitates tumor lysis by different subsets of effector lymphocytes.

Fig. 8. RA treatment does not affect MHC class I-restricted peptide-specific lysis of "responder" neuroblastoma cells by CD8+ CTLs. The HLA-A11+ neuroblastoma cell lines SK-N-BE (A) and SK-N-FI (B), either untreated (□) or treated with RA (■) for 96 h were prepulsed with the IVT peptide at the indicated concentrations and tested for sensitivity to lysis by the peptide-specific HLA-A11-restricted CD8+ CTL clone BK289 in a standard 4 h 51Cr release assay at 2:1 E:T ratio. Data from one of two performed experiments.

Fig. 9. Bystander T cell-mediated cytolysis of neuroblastoma cells treated with RA is higher than that of untreated tumors. SK-N-SH (A) and MC-IXC (B) cells either untreated (□) or treated with RA (■) were cocultured with activated CTLs in a 16 h 51Cr release assay at 5:1 and 2.5:1 E:T ratios. One representative of three experiments.

Fig. 10. Effect of RA treatment on killing of neuroblastoma by NK cells. SK-N-SH cells either untreated (□) or treated with RA (■) and K562 cell line (○) were used as targets for the NK lymphoma cell line Nishi (A) or polyclonal NK cultures established from healthy donor (B) in a standard 4 h 51Cr release assay. One representative experiment is shown in the figure.
The response rates in human neuroblastomas are low with available therapeutic modalities. Our findings suggest that the combined application of RA and T cell-based immunotherapy may be a promising and effective combination for the treatment of neuroblastoma.

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Retinoids Act as Multistep Modulators of the Major Histocompatibility Class I Presentation Pathway and Sensitize Neuroblastomas to Cytotoxic Lymphocytes

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