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Control of Metastasized Pancreatic Carcinomas in SCID/Beige Mice with Human IL-2/TKD-Activated NK Cells

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Pancreatic carcinoma, the fifth leading cause of cancer-related mortality, frequently presents the stress-inducible heat shock protein 70 (Hsp70) on the cell membrane. Therefore, we explored an immunological approach exploiting the efficacy of NK cells activated either with low dose IL-2 plus Hsp70-peptide TKDNNLLGRFELSG (TKD; IL-2/TKD) or with IL-2 alone in a xenograft pancreatic carcinoma model. An orthotopic injection of either 2.5 x 10⁶ or 1 x 10⁶ Colo357 cells in SCID/beige mice resulted in rapidly growing primary tumors and the development of hepatic metastases on days 5 and 10, respectively. In line with results of in vitro migration assays, these NK cells also had the capacity to infiltrate pancreatic tumors and liver metastases in tumor-bearing mice. In vitro, a combined treatment of NK cells with IL-2/TKD but neither of the two treatments alone causes a profound increase in the lytic capacity against Hsp70 membrane-positive Colo357 cells. In vivo, a single i.v. injection of these NK cells on day 15 post-tumor inoculation resulted in a significant reduction in tumor weights, a delayed onset of hepatic metastases, and a prolonged life expectancy. In contrast, identically treated T cells and NK cells treated with IL-2 alone were significantly less efficient in controlling pancreatic tumors and metastases. Most importantly, four repeated i.v. infusions of IL-2/TKD-activated NK cells eradicated primary tumors and prevented hepatic metastases. In summary, our mouse data have implicated that NK cells pre-activated with IL-2/TKD might provide a novel therapeutic tool for the treatment of aggressive, Hsp70-positive pancreatic carcinoma.

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6 Abbreviations used in this paper: o.t., orthotopic; Hsp, heat shock protein; TKD, 14-mer peptide TKDNNLLGRFELSG; Hsc 70, constitutive Hsp 70.
NK cells, but not of identically treated CD3⁺ T cells or IL-2-activated NK cells, resulted in a significant reduction in the weight of primary pancreatic carcinoma, a delayed onset of metastatic dissemination, and a significantly prolonged life expectancy. Furthermore, four repeated injections of IL-2/TKD-activated NK cells eradicated primary tumors and hepatic metastases.

Materials and Methods

Animals

Female and male C.B.-17/krHsd-scid-bg (SCID/beige) mice were obtained from an animal breeding colony (Harlan Winkelmann) and maintained in pathogen-free, individually ventilated cages (Tecniplast). The animals were fed sterilized, laboratory rodent diet (Meika) and used for experiments at 6–12 wk of age. All animal experiments had been approved by the Regierung der Oberpfalz and were in accordance with institutional guidelines.

Pancreatic cancer cell line

After three i.p. passages through SCID/beige mice, a master cell bank (total, 1 × 10⁶ cells) was established from the human pancreatic adenocarcinoma cell line Colo357, provided by Dr. H. Graf (Scheren; Ref. 32). Identical aliquots (5 × 10⁶) of this fast-growing (doubling time increased from 40 to 24 h) and highly metastatic variant were frozen in liquid nitrogen. After thawing, Colo357 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin) at 37°C in 5% CO₂. Single-cell suspensions were derived by short-term (1<1 min) trypsin-EDTA treatment (all cell culture reagents were purchased from Life Technologies). Before injection viability of Colo357 cells, as determined by trypan blue exclusion assay, was always >95%.

Implantation of Colo357 cells o.t.

For o.t. implantation, mice were anesthetized by i.p. injection of 0.03–0.04 ml of xylazine (6–8 mg/kg body weight; WDT) and ketamine (90–120 mg/kg body weight; WDT). The abdomen was sheared and sterilized with 2-isopropanol (70%). The skin and peritoneum were opened by a 1-cm incision along the linea alba caudal of the processus xiphoideus under strict aseptic conditions. The o.t. injection of either 25 or 10 μl of the Colo357 stock solution (100 × 10⁶ cells/ml) into the exteriorized pancreatic corpus using a 25-μl glass syringe with a 22-gauge needle (Hamilton) was visually controlled using a 7× stereomicroscope (Zeiss). After replacement of the pancreas into the abdominal cavity, the incision was closed in two layers using a 6-0 vicryl suture for the peritoneum and 7.5-mm suture clips for the skin (Fine Science Tools). This mouse model was found to be technically standardized and feasible.

Flow cytometry

Hsp70 membrane expression was routinely determined on Colo357 cells derived from cell cultures before injection, from single-cell suspensions of random tumor samples, and from patient-derived pancreatic tumor samples by flow cytometry, using a FITC-labeled mAb directed against the extracellular exposed epitope of membrane localized Hsp70 (mHsp70;1; multimime). Briefly, after incubation of single-cell suspensions of viable tumor cells (0.2 × 10⁶ cells) with Hsp70-specific mAb for 30 min on ice and following two washing steps, nonfixed cells were analyzed on a FACSCalibur instrument (BD Biosciences). Isotype-matched control Abs and a human HLA class-I-specific mAb (clone W6/32) were used as negative and positive controls.

Separation and stimulation of effector cells

CD3⁺ CD94⁺ NK cells were selected from leukapheresis products obtained from healthy human volunteers using either CD94-biotin mAb (HP3-D9; Ancell Immunology Research Products) and anti-biotin magnetic microbeads (Milenyi Biotec) or following a standard CD3/CD19 depletion protocol (Miltenyi Biotec), as described earlier (33). The purity of NK cells as determined by flow cytometry using directly conjugated CD3-FTTC/ CD16/56-PE (BD Biosciences) and CD94-FTTC (Ancell) mAbs on day 2 after separation and stimulation was always >95%. Identical numbers (2 × 10² cells/ml) of CD3⁺ CD94⁺ NK and CD3⁺/CD94⁻ T cells were incubated separately in RPMI supplemented with low dose IL-2 (100 IU/ml) plus TKD (2 μg/ml) (Bachem) or with IL-2 (100 IU/ml) alone for 4 days. TKD is a GMP grade 14-mer peptide of the C-terminal substrate-binding domain of human Hsp70 (TKDNLNLGRFELSG, aa 450–463) known to exert immunostimulatory activity on NK cells (25).

Chromium release assay

The cytolytic activity of CD3⁺ CD94⁺ NK cells incubated with Hsp70 peptide TKD (2 μg/ml), with IL-2 (100 IU/ml) alone, or with a combination of IL-2/TKD for 4 days was determined in a standard ⁵¹Cr release assay (34) at E:T ratios ranging from 40:1 to 5:1. Cultured single-cell suspensions derived from Colo357 tumors that were surgically removed from SCID/beige mice on day 22 after tumor cell inoculation were used as target cells.

Adaptive transfer of preactivated NK and T cells

On day 4 after stimulation, both IL-2 and TKD were removed by intensive washing. Cells were then resuspended in RPMI at a cell density of 30 × 10⁶ cells/ml; 100 μl of this stock solution containing 3 × 10⁶ cells/mouse were injected i.v. into the tail vein of tumor-bearng mice at indicated time points. For repeated injections, activated NK or T cells were derived from one leukapheresis donor, but different animals received NK or T cells derived from different donors. Effector cells were injected into tumor-bearing SCID/beige mice only if viability was >95%.

Autopsy

After o.t. implantation of tumor cells, immunodeficient control mice and immunoreconstituted mice were sacrificed by craniofacial dislocation. The weight of the mice was then determined, the peritoneal cavity was inspected morphologically, and primary tumors and livers were excised in total. The weight of each primary pancreatic tumor was measured separately. Organs were kept on ice-cold, sterile PBS until needed for further investigations.

Histochemistry and immunohistochemistry

After determination of the weight, parts of the collected tumors and mouse livers, which were cut in equal pieces 4-mm thick, were fixed in Bouin’s solution containing 71.5% picric acid, 23.8% formaldehyde, and 4.7% acetic acid and embedded in paraffin. Consecutive section pairs of the liver and tumors (5 μm) were prepared from the ventral margin of each piece at a distance of 250 μm. The morphology of tumors and organs was visualized by standard H&E and Masson-Goldner staining; nuclei were costained in 1% Mayer’s hematoxylin (Dako).

For immunohistochemistry, the activity of the endogenous peroxidase was blocked with freshly prepared 1% hydrogen peroxide containing 0.1% sodium azide. Sections were then incubated with unconjugated anti-human Hsp70/constitutive Hsp70 (Hsc70) (rat; clone 7F4; provided by Dr. Elisabeth Kremmer, GSF-Institute of Molecular Immunology, Munich, Germany) at 4°C overnight. For the detection of effector cells, sections were always heated for 30 min at 97°C and then incubated with anti-human CD94 (mouse; clone DX-22; IgG1; chioscience), anti-human CD3 (mouse; clone OKT3; Ortho Biotech) or an IgG1 isotype-matched control (mouse; IgG1; Dako) mAbs as described before. After a washing, a rabbit anti-rat or rabbit anti-mouse HRP-conjugated secondary Ab (Dako) was incubated for 2 h at room temperature. Diaminobenzidine (Dako) was used as a chromogen. Sections were counterstained with 1% Mayer’s hematoxylin (Dako/Cyto- mation) for 30 s and analyzed on an Axiovert 25 microscope (Zeiss).

Statistics

The significance of the data was determined by using a nonparametric rank sum test (Mann-Whitney).

Results

In vivo tumor growth of o.t. transplanted Colo357 cells

An o.t. injection of 2.5 × 10⁶ Colo357 pancreatic carcinoma cells suspended in 25 μl of medium resulted in aggressive, rapidly growing pancreatic tumors (Fig. 1A). On day 30, the average tumor weight was 0.60 ± 0.14 g (n = 6); and due to cachexia, stenosis of the proximal ileum, and/or ductus choledochus, the animals rapidly became moribund. After injection of 1 × 10⁶ Colo357 cells, the average tumor weight on day 30 was 0.20 ± 0.08 g (n = 15), and first signs of disease were detectable from day 50 onwards with an average tumor weight of 0.43 ± 0.09 g (n = 6; Fig. 1A). Tumor take at any tested time point, for both injection modes was always 100%. A comparative phenotyping of Colo357 cells from tissue culture and from single-cell suspensions derived from tumor-bearing mice on days 15, 22, and 30 revealed no significant
differences in their Hsp70 membrane expression pattern; on average 60 ± 9.8% of the Colo357 cells were Hsp70 membrane-positive, as determined on at least three tumor biopsies at each time point. Single-cell suspensions of these Hsp70-expressing Colo357 cells were used as targets in a standard chromium release assay. As summarized in Fig. 1B, Colo357 cells were lysed most efficiently by CD3−CD94− NK cells that had been treated with low dose IL-2 plus TKD (IL-2/TKD) for 4 days. None of the two reagents alone exhibited a comparable stimulatory capacity on NK cells (Fig. 1B). IL-2/TKD-activated T cells did not exhibit significant lysis of Colo357 cells (data not shown).

Adoptive transfer of IL-2/TKD-activated effector cells

In vitro, the combined treatment of NK cells with IL-2/TKD was a prerequisite for the initiation of the migratory capacity toward Hsp70 membrane-positive tumors (21). With regard to these results, the question arose whether these NK cells were also attracted to Hsp70 membrane-positive tumors (21). With regard to these re-

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showing first signs of disease by day 20 and all animals dying from progressive tumor disease on day 42. In contrast, a single injection of IL-2/TKD-activated NK cells significantly prolonged the survival of the mice, with \(60\%\) of the mice still alive on day 72 (Fig. 2D). As already shown earlier, IL-2 alone was less efficient in the stimulation of an Hsp70-mediated antitumor immune response in NK cells; on day 52, all mice died from progressive tumor disease (Fig. 2D).

**Effects of NK cells on hepatic metastases**

Because pancreatic tumors in humans have a high potential for hepatic metastases, mouse livers were inspected by immunohistochemistry using the rat anti-human Hsp70/Hsc70-specific mAb 7F4. The injection protocol was identical with that shown in Fig. 2A. As expected, the anti-human specific mAb did not stain normal mouse liver on day 0 preceding tumor cell inoculation (Fig. 3A).

However, by day 22 after o.t. transplantation of \(2.5 \times 10^6\) human Colo357 pancreatic carcinoma cells, liver metastases had developed, and large areas of the mouse liver stained positive for human Hsp70-reactive NK cells. Arrows mark the localization of CD94\(^+\) NK cells. Consecutive sections incubated with a mouse anti-human CD3-specific mAb revealed no staining (data not shown). Representative photographs of resected pancreatic tumors derived from SCID/beige mice on day 22. Tumor-bearing animals were injected i.v. with medium (control), with IL-2/TKD-activated T or NK cells, or with IL-2-activated NK cells on day 15 (upper graph). The tumor weight of each individual tumor was given below the photographs. The average tumor weights of three to five independent experiments \(\pm\) SD were shown below. Reduction in tumor weight was statistical significant between control tumors and tumors of mice injected with IL-2/TKD-activated NK cells \((p < 0.01)\), but not between control tumors and tumors of mice injected with either IL-2/TKD-activated T cells or IL-2-activated NK cells \((p < 0.1)\). D, Overall survival of tumor-bearing SCID/beige mice after o.t. injection of Colo357 cells and a single i.v. injection either with medium (white squares), IL-2/TKD-activated T (light gray squares), IL-2/TKD-activated NK cells (black crossed squares), or IL-2-activated NK cells (dark gray squares) on day 15. The increased survival of mice injected with IL-2/TKD-activated NK cells was significant \((p < 0.01)\) if compared with control mice.
immunohistochemically defined metastases was counted in liver sections of SCID/beige mice. For this purpose, the left lateral lobe of the liver was resected on days 5, 10, 15, 20, 25, and 30 after o.t. injection of 2.5 × 10^6 Colo357 cells and on days 10, 20, 30, and 40 after injection of 1 × 10^6 Colo357 cells. Each organ was cut into three identical parts to prepare three consecutive section pairs. The size of the counted metastases in the liver ranged between 50 and 4000 μm after o.t. injection of 2.5 × 10^6 Colo357 cells (Fig. 3B) and between 20 and 2500 μm after o.t. injection of 1 × 10^6 Colo357 cells (Fig. 3C) after staining with the human-specific mAb 7F4. Hepatic metastases were initially detected by day 5 after inoculation of 2.5 × 10^6 Colo357 cells (Fig. 3B) and by day 10 after inoculation of 1 × 10^6 Colo357 cells (Fig. 3C). On day 30, immunodeficient mice contained on average 4.8 ± 3.5 and 4.1 ± 2.8 liver metastases after injection of 2.5 × 10^6 and 1 × 10^6 Colo357 cells, respectively. The adoptive transfer of preactivated T cells on day 15 exhibited no significant inhibitory effect on the development of distant metastases in the liver compared with immunodeficient control animals. In contrast, a single dose of preactivated NK cells on day 15 resulted in a significant reduction of the number of metastases in the liver on days 20, 30, and 40 (Fig. 3, B and C, p < 0.01). The number of examined mouse livers at each indicated time point ranged from 10 to 13.

Repeated infusions of effector cells

To further improve the antitumor activity, mice were repeatedly injected up to four times with effector cells. After o.t. injection of 1 × 10^6 Colo357 cells, mice were reconstituted with either 3 × 10^6 IL-2/TKD-activated T or NK cells once on day 15; twice on days 15 and 25; three times on days 15, 25, and 35; or four times on days 15, 25, 35, and 45 (Fig. 4A). Separated T or NK effector cells for repeated injections of one mouse were obtained from one leukapheresis donor; different mice were treated with T or NK cells derived from leukapheresis products derived from different human donors. Every 10 days after the last effector cell infusion, mice were sacrificed and inspected for primary pancreatic tumors and hepatic metastases. As summarized in Fig. 4B, three to four repeated NK cell infusions into the tail vein of tumor-bearing SCID/beige mice significantly reduced the weight of primary tumors. The minimal tumor load after the fourth injection round of NK cells was completely necrotic, as determined by histological analysis of H&E-stained tumor sections (data not shown). This finding was further supported by the fact that these mice did not develop recurrent disease. Immunohistological examinations of the liver using the human-specific Ab 7F4 revealed no metastatic dissemination in this organ on day 55 (data not shown). In contrast, the reduction of the tumor weights using IL-2/TKD-activated T cells was less pronounced after one to three injection cycles. On day 55, 10 days after the fourth i.v. injection of T cells, tumors showed progressive growth kinetics comparable with that observed in control animals. All control and T cell-treated mice died from progressive tumor disease between days 55 and 60 (data not shown).

Similar results were obtained in mice that had been treated o.t. with 2.5 × 10^6 Colo357 cells and three repeated effector cell injections. However, due to the very rapid tumor growth, T and NK

Table I. NK and T cell counts in Colo357 tumor sections on days 22 and 27

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<tr>
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<th>d22</th>
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<tbody>
<tr>
<td>NK cells</td>
<td>40 ± 14 (n = 6)</td>
<td>136 ± 79 (n = 8)</td>
</tr>
<tr>
<td>T cells</td>
<td>6.7 ± 9.4 (n = 6)</td>
<td>20.3 ± 8.5 (n = 8)</td>
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*SCID/beige mice were injected (o.t.) with Colo357 tumor cells (2.5 × 10^6) on day 0 and immunoreconstituted either with 3 × 10^6 IL-2/TKD-activated T cells or NK cells on day 15. Mice were sacrificed on days 22 and 27, and consecutive tumor sections (5 μm) were examined immunohistochemically using Abs directed against CD3, CD94, and isotype-matched control Abs. Results indicate the number of CD3⁺CD94⁺ NK cells and CD3⁺ T cells within a defined tumor region of 1 cm² derived from 6–8 independent experiments ± SD.

p < 0.01.
cells were given on a weekly basis. The experiment was terminated after three injections cycles because all control and T cell-treated animals were dead on day 36 (data not shown).

Discussion

NK cells have been found to play a crucial role in the control of hematological malignancies (35). As previously described by our group, preactivation of CD3−CD94+ NK cells with IL-2 plus Hsp70 peptide TKD resulted in an enhanced cytolytic and migratory capacity toward Hsp70 membrane-positive tumor cells in vitro (25). Human pancreatic carcinomas, the fifth leading cause of cancer-related mortality, were frequently found to be Hsp70 membrane-positive (unpublished observation) and thus were susceptible to NK cell-mediated lysis in vitro (15). In this study, we addressed the question of whether Hsp70-reactive NK cells have the capacity to eradicate primary tumors and distant metastases originating from Colo357 cells. These tumors exhibited an Hsp70 cell surface-positive phenotype on ~60% of the cells (21). As early as 1 wk after adoptive transfer of IL-2/TKD-activated NK cells, weights of primary tumors were significantly reduced. In contrast, identically treated T cells and NK cells stimulated with IL-2 alone showed only a moderate reduction in the mass of primary Colo357 tumors.

Interestingly, a single i.v. injection of IL-2/TKD-activated NK cells not only inhibited primary tumor growth within the first week after injection but also significantly prolonged the life expectancy of tumor-bearing mice. These data indicated that even in immunodeficient SCID/beige mice lacking functional T and B cells but also IL-15, which is required for the NK cell development, human NK cells exerted their protective ability for an extended period of time. Again the Hsp70 peptide TKD was a prerequisite for an efficient stimulation of an antitumor immune response in NK cells.

It has been shown by others that NK cells play important roles in the control of distant metastases in animal models and in humans (27–31). In the present study, we observed an inhibitory effect of IL-2/TKD-activated NK cells on the metastatic spread in the liver when compared with control and T cell-treated animals. Furthermore, metastasis-free survival rates could be correlated with an enhanced NK cell activity in a clinical phase I trial (28).

NK cell-treated mice had a later onset of severe signs of tumor disease compared with control mice or mice injected with T cells. These data also suggested that the partial immunoreconstitution with IL-2/TKD-activated NK cells has a life-prolonging effect. With regard to recently published data (39), studies are ongoing analyzing as to whether the addition of human DCs and T cells might further improve the antitumor effects in our xenograft mouse model.

To evaluate the relevant dose of NK cells necessary for a complete tumor control the infusions of IL-2/TKD-activated NK cells were repeated. After four injection cycles, primary tumors as well as distant metastases were eliminated. In contrast, repeated T cell
injections controlled primary tumors only until day 45; then tumors grew progressively. These findings might be explained in part by the fact that NK cells have higher intracellular glutathione levels than do T cells (40), which might enable a prolonged survival and an enhanced cytolytic activity of NK cells in immunodeficient mice. In summary, our data provide evidence that even in IL-15-deficient hosts IL-2/TKD-activated NK cells control primary pancreatic tumors and hepatic metastases. In a recently published clinical phase I trial, the tolerability, feasibility, and safety of IL-2/TKD-activated NK cells were tested in patients with colorectal and lung carcinoma. Apart from an excellent safety profile of these effector cells, we were able to successfully demonstrate that repeated treatment cycles resulted in clinical responses even in multiply metastasized patients, refractory to standard chemo/radiotherapy (41). Again, low dose IL-2 alone was inefficient in stimulating the Hsp70-reactivity in patient-derived NK cells.

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Disclosures
The authors have no financial conflict of interest.

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