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Protitumorska učinkovitost intrahepatičnih naravnih celic ubijalk T pri živalih

Antitumor Efficancy of Intrahepatic Natural Killer T Cells in Animal Models

IZVLEČEK

KLJUČNE BESEDE: ubijalke naravne, novotvorbe – imunologija

Naravne celice ubijalke T (NKT), ki izražajo invariantni T-celični receptor in označevalce celične vrste NK (NK1.1 in IL-2Rβ), so v zadnjih letih postale pomemben predmet raziskovanja, saj so očitno povezane z naravno odpornostjo za tumorje, nekatere povzročitelje okužb in za nastanek avtoimunosti. Kako te celice delujejo, še ni znano. Ve pa se že, da je njihovo delovanje omejeno s CD1, saj se vežejo z glikolipidnimi ligandi, proteini vročinskega šoka in dugimi dejavniki, povezanimi s stresom. Verjetno je, da so pomembne za spoznavo in prenos informacij, ki nastanejo ob nastanku tumorjev ali pa ob poškodbi telesnih celic. Posredno lahko sprožijo dejavnost imunskega sistema ali neposredno zavrejo tumorsko rast. Nadalje lahko izdelujejo različne citokine, ki vplivajo na ravnovesje med celicami TH1 in TH2.

Celice NKT zelo verjetno nastajajo tudi zunaj priželjca, najverjetneje v jetrih. V naši raziskavi smo ugotovili, da pri miših nastanek jetrnih celic NKT sprožijo:

a) delna hepatektomija (pHx),
b) vbrizganje streptozotocina (sredstvo, ki izzove nastanek avtoimunskega diabetesa) in
c) vbrizganje peptidoglycan monomera – PGM in PGM-Zn, ki ju pripravljamo iz po Gramu pozitivnih bakterij.

V navedenih poskusih smo ugotovili, da so pravkar osamljene MNLC iz jeter in vranice za celice YAC-1 in singenske timocite močno ubijalske, kar spodbuja domnevo, da so avtoreaktivne jetrne celice NKT zelo pomembne pri uravnavanju protitumorske imunosti in za razmnoževanje epitelijskih celic.

ABSTRACT

KEY WORDS: killer cells natural, neoplasms – immunology

Natural killer T (NKT) cells, which co-express the invariant T cell receptor and markers of NK cells lineage (NK1.1 and IL-2Rβ) have become a major focus in the studies of the innate immune response to tumors and infectious diseases, as well as in autoimmunity, but the precise immunological function of these »primitive« T lymphocytes is still not well defined. As

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CD1-restricted cells they interact with glycolipid ligands, heat shock proteins and other »stress« or »danger« signals produced by tumors or injured self cells, transferring important information to the rest of the immune system or directly suppressing the tumor growth. Furthermore, depending on the stimulus, they produce various immunomodulatory cytokines, affecting the TH1/TH2 immune balance.

Since particularly the liver might be a site of extrathymic generation of the NKT cells, we demonstrate herein evidence that in mice these cells might be induced by: a) partial hepatectomy (pHx), b) injections of streptozotocin (provoking autoimmune diabetes) and c) injections of peptidoglycan-monomer-PGM and PGM-Zn, prepared from Gram + bacteria. The data also showed that in all experimental models freshly isolated hepatic and particularly splenic MNLC became markedly cytotoxic to YAC-1 cells, as well as to syngeneic thymocytes, suggesting that autoreactive NKT cells in the liver might be key players in the regulation of anti-tumor immunity and epithelial cell proliferation.

INTRODUCTION

Cells of the innate immune system have not only the responsibility of surveying and »informing« the host of a breach in integrity, but also intrinsic antitumor effector functions, including lysis of tumor cells and the production of cytokines that inhibit tumor growth and block angiogenesis. NK, NKT and gamma delta T cells express activating receptors such as NKG2D that recognize MHC class I chain-related (MIC) or ULI6-binding proteins that become upregulated on tumor cells, while some NKT cells and gamma delta T cells also express T cell receptors with restricted diversity that recognize tumors through lipid or protein antigens presented in the context of MHC proteins *(1)*. Therefore, cells of innate immune system respond to »danger« signals, which can be provided by growing tumors as a consequence of the genotoxic stress of cell transformation and disruption of the surrounding microenvironment *(2)*. Under ideal conditions these signals will induce inflammation, activate innate effector cells with antitumor activity and stimulate professional antigen-presenting cells (APS) to engulf tumor-derived antigens and migrate to draining lymph nodes to trigger an adaptive response by T and B lymphocytes. However, this well-orchestrated surveillance operation is often ineffective, since progressing tumors exhibit various mechanisms that promote evasion from immune recognition *(3)*. The strategies which induce the proliferation of innate cells or APC or harnessing of innate immune cells as effectors therefore represent the current challenges in cancer immunotherapy *(1)*.

In this context, it was postulated that the adult liver of mice and humans might be a crucial organ in the first line of host defence against bacterial infections and hematogenous tumor metastases, since it contains a unique phenotypic distribution of Kupffer cells, NK cells and NKT cells responses. The later, NKT cells, are a highly conserved subset of primitive lymphocytes, which in phylogenetic sense stand between the NK and T cells and bear the surface receptors of both cell types *(4–7)*. Most of them constitutively express IL-2Rβ-chain (IL-2β) and have lower surface density of αβ T cell receptor (or CD3) (termed intermediate TRC; TCR^{int}) *(4–9)*. Their ontogeny is disputed, but $NK1.1^{+}$ $CD3^{int}CD4^{low}$ T cells might be generated by an alternative intrathymic pathway without passing through the double-positive $CD4^{+}8^{+}$ stage, as well as extrathymically in multiple sites in the body (liver, uterus, small intestine, salivary gland) *(6, 7)*. Both pathways are totally repressed in normal euthymic mice and became active only in the cases of severe T depletion. More importantly, these non-traditional T cells are characterized by restricted TCR repertoire, having the unusual property of being readily stimulated by non-protein components of microorganisms and some self-antigens, presented in the context with monomorphic MHC class I antigens, such as CD1 and TL in mice, and non-classical HLA class I antigens in humans (HLA-E, -F and -G) *(3–9)*. Recognizing

various non-peptide antigens, like lipid and glycolipid components of bacterial cell walls, alpha galactosylceramide, metabolites produced by the cells, or stress proteins MICA and MICB, NKT cells and CD1-restricted γδ T cells therefore appear to be able to identify the abnormal self-cells, as well as the type of infection at an early stage, responding to these stimuli by activation of potent cytolytic activity (NK, LAK and TRC mediated). Furthermore, since NK1.1 antigen expressed on TCR^{int} population of primitive lymphocytes usually defines a subset of cells that produce very high titers of cytokines, while the stimulation which goes through TCR results in the higher production of IL-4, the stimulation of NKT cells is also responsible for the TH1/TH2 immune balance *(6–9)*.

Since particularly the liver might be a site of extrathymic generation of the NKT cells, in this study we attempted to elucidate the phenotypic and functional properties of intrahepatic and splenic mononuclear lymphoid cells (MNLC) in experimental models of: a) tissue regeneration induced by pHx, b) autoimmune diabetes mellitus induced by injections of streptozotocin and c) bacterial exposure induced by injections of peptidoglycan-monomer-PGM and PGM-Zn, prepared from Gram + bacteria. In all models, augmented proportions of NKT cells were found in the liver, while freshly isolated hepatic and splenic MNLC became markedly cytotoxic to YAC-1 cells, as well as to syngeneic thymocytes, suggesting that autoreactive NKT cells in the liver might be key players in the regulation of antitumor immunity and epithelial cell proliferation.

MATERIAL AND METHODS

Mice

Experiments were done on C57Bl/6 and CBA mice, provided by animal cure facility of Medical School Rijeka.

Partial hepatectomy

Mice were under ether anesthesia subjected to 1/3 pHx, after removing the median liver lobe. Animals suffered bleeding on first, second and seventh day after pHx.

Induction of autoimmune diabetes mellitus

Genetically susceptible to CBA, male mice were injected by low doses of streptozotocin for five days (40 mg/kg of body weight). The animals died on the 18th day, when maximal hyperglycemia was seen.

TREATMENT WITH PGM-ZN

As previously reported *(10)* peptidoglycan monomer (PGM)-(GlcNAc-MurNAc-L-Ala-D--iso-Gln-meso-diamminopimelic acid (w-NH2)--D-Ala-D-Ala) and PGM linked with zinc (PGM-Zn) (Pliva, Zagreb, Croatia) were prepared by biosynthesis from the culture fluids of *Brevibacterium divaricatum* NRRL-2311. They were dissolved in PBS and injected every second day (10 mg/kg of body weight, intraperitoneally (i. p.) for 6 days (total dose 30 mg/kg). Mice in the control groups were treated with the same volume of PBS. Two days after the last injection, the animals were put down to isolate the hepatic and splenic MNLC.

Isolation of hepatic and splenic MNLC

MNLC were isolated from intact or regenerating liver, using a modification of the method of Seglen as we previously described *(10)*. A single suspension of spleen MNLCs was prepared in RPMI 1640 medium (Gipco BRL, Basel, Switzerland), after elimination of erythrocytes by lysing solution.

Immunofluorescent staining and flow cytometry

The surface phenotypes of intrahepatic MNLC and splenocytes were identified by direct immunofluorescence analysis on FACScan (Becton Dickinson, Immunocytometry Systems, Mountain View, CA), using primary fluorescein isothiocyanate-conjugated anti-CD8 and anti-CD3 and phycoerythrin-conjugated anti-CD4, anti-NK-1.1, anti-CD122 (IL-2Rβ) and anti-TCR γδ mAbs purchased from Becton Dickinson Co (Mountain View, CA, USA). All samples had adequate isotypic controls. Propidium iodide (Sigma, MO) (1 mg/ml) stained dead cells were excluded by electronic gating.

Flow-cytometry cytotoxicity assay

The cytotoxicity of freshly isolated hepatic and splenic MNLC was tested against YAC-1 (a Moloney virus-induced lymphoma), as well as against the syngeneic thymocytes. Tests were made by the use of flow cytometry and PKH-26 Red Fluorescent Cell Linker Kit and 2h-cytotoxicity assay (Sigma Biosciences, St Louis, MO).

Statistical analysis

Data were analysed using the Sigma Plot Scientific Graphing System, Version 6.10. Linear regression equations were generated using a least-squares method and analysed for differences of covariance. Statistical significance was calculated by Mann Whitney U test. The differences were considered significant for $p < 0.05$.

RESULTS

NKT cells are overexpressed in regenerating liver, as well as in the livers of mice treated with streptozotocin or PGM-Zn

As presented on Figure 1, in all experimental models the proportion of cells with NKT phenotype ($CD3^{int+}/NK1.1^{+}$ cells or $IL2R\beta^{+}CD3^{int}$ cells) in the liver increased, which was not seen in the spleen (not shown). Most of them were expressing a very early activation marker CD69 (not shown).

Cytotoxic activities of hepatic and splenic MNLC against NK-sensitive and autologous targets are increased after partial hepatectomy, as well as after treatment of mice with streptozotocin and PGM-Zn

Immediately after isolation, freshly isolated hepatic and splenic MNLC were tested against YAC-1 cell line and syngeneic thymocytes. The data showed that in all models hepatic and splenic effectors acquired a high cytotoxicity against a NK cells sensitive target (Figure 2), as well as against the autologous cells (not shown), suggesting that accumulated NKT cells were autoreactive cells, which subsequently contributed to activation of classical NK cells. Moreover, in some models (treatment with PGM-Zn) a highly significant positive correlation was found between the proportion of NKT in the liver and cytotoxic activities of hepatic and splenic MNLC against YAC-1 and syngeneic targets ($r = 0,95$ and $r = 0,88$ respectively; $p < 0.01$).

DISCUSSION

The data clearly show that conditions such as liver regeneration, autoimmune disease and exposure to bacterial toxins might elevate the number and function of extrathymic T cells, which are generated or accumulated mainly in the liver. Moreover, since in all models freshly isolated hepatic and splenic MNLC became markedly cytotoxic to YAC-1 cells as well as to syngeneic thymocytes, the data confirm that various danger-related signals might induce the antitumor activity of NK cells through activation of autoreactive hepatic NKT cells *(4–9)*. The hypothesis is consistent with the »danger« and »integrity« models, which hold that immune response might be triggered by endogenous alarm signals coming from the stressed or injured tissue *(2, 11)*, as well as with recent knowledge about the function of heat-shock protein (HSP)-chaperoned peptides derived from damaged tissue *(12)*. Moreover, the data showing the enhancing effect of PGM-Zn NKT induction and cytotoxicity are consistent with the »pattern recognition receptor« model, which shows that manipulation of the innate immune response might occur when the dendritic cells detect the exogenous infectious agents through Toll-like receptors *(1,13,14)*. Thus, although the activation pathways of hepatic NKT and possible mechanisms by which these effectors contribute to antitumor effects might be different in each of our experimental models, we would like to emphasize that activation of primitive lymphocytes in the liver might be a promising strategy in cancer immunotherapy.

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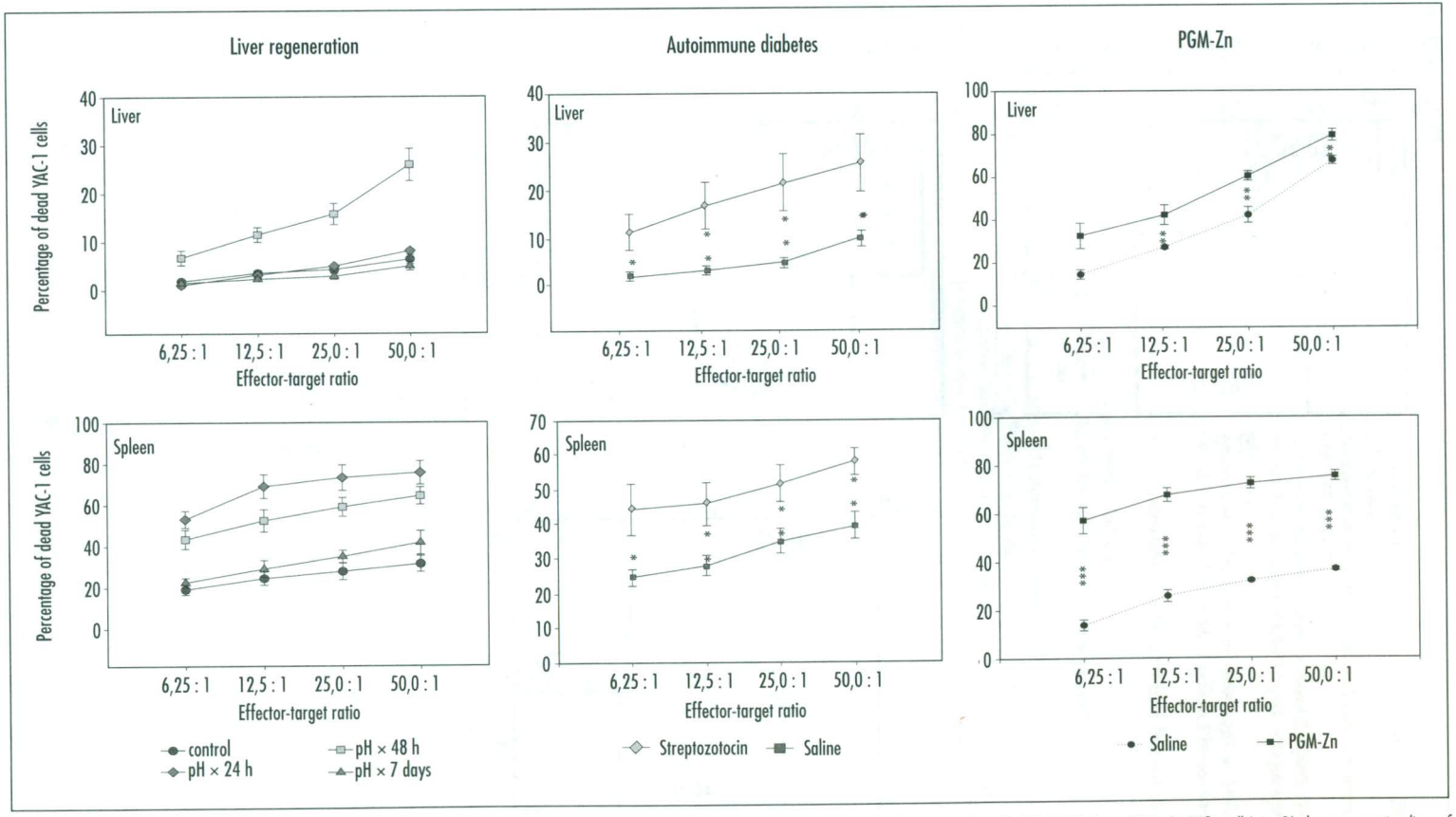

Figure 1. *Flow cytometric detection of NK cells ($CD3^-NK1.1^+$ or $CD3^-/IL\text{-}2R\beta^+$ cells), NKT cells ($CD3^{intermediate}NK1.1^+$ or $CD3^{intermediate}/IL\text{-}2R\beta^+$ cells) and T cells ($CD3^+NK1.1^-$ or $CD3^+/IL\text{-}2R\beta^-$ cells) in: 1) the regenerating liver of C56Bl/6 mice 48h after pHx, 2) diabetic CBA mice treated with streptozotocin at the time of maximal hyperglycemia (number in parenthesis represents control values for NKT cells) and 3) C56Bl/6 mice treated with PGM-Zn.*

Figure 2. *Cytotoxic activities of freshly isolated hepatic and splenic MNLC against NK-sensitive target (YAC-1). Effector cells were isolated from: 1) partially hepatectomized mice 1, 2 and 7 days after pHx, 2) diabetic mice and 3) mice treated in vivo by PGM-Zn. Results are presented as mean ± standard error of 6 experiments, made in triplicate. *p<0.05; **p<0.01.*

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