Evaluation of major histocompatibility complex (MHC) class I and activating natural killer (NK) cell ligands expression of uveal melanomacell lines

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Abstract

Immunophenotype expression analysis of primary uveal melanoma cell lines OCM1, OM431 and liver uveal metastasis cell lines OMM2.3, OMM1, was done for MHC class I by staining using either specific mAbs for HLA-A2, HLA-BC, or a pan HLA-class I mAb. However, comparative phenotypic expression changing of NK cell activating receptor ligands, especially DNAM-1 ligands (PVR and nectin-2) during liver metastasis, was done and correlated with susceptibility to NK cell cytotoxicity evaluating by flow cytometry.

All examined cell lines expressed low level of MHC class I in comparison with healthy PBLs that could be of great relevance for the metastatic potential of uveal melanoma. While they expressed NK cell activating DNAM-1 ligands that correlated with observed their high susceptibility to NK cell mediated lysis in a feature similar to skin melanoma.The findings are compatible with a potential role of NK cell mediated control of hematogenic metastatic spread that appears less than as in lymphatic compartment.The results demonstrate stage-dependent expression of DNAM1 ligands in uvealmelanoma in addition to predictable MHC class I reduction with increasing in their susceptibility to NK cell lysis during disease progression.

Keywords: DNAM-1ligands, MHC class I, Metastasis, NK cells, Uveal melanoma

Introduction

Uveal melanoma is ocular melanoma slowly develops from the pigmented cells of the choroid (choroidal melanoma), but it also can develop from the pigmented cells of the iris (iris melanoma) that residue within the uvea of the eye.

Although, both uveal and skin melanoma tumors are derived from melano-cytes but the unique structure and function of melanocytes within the choroid of the eye make them differ with respect to aetiology, metastatic behavior and immune biology. Therefore, they are clinically and genetically distinct (Yanoff & Sassani, 2009)

Uveal melanoma is the most common primary intraocular tumor of the eye. Approximately 50% patients with large primary tumors have liver metastases. Despite its lower incidence (0.47–0.79 new cases per 100000 individuals) compared to cutaneous melanoma (Albert *et al.*, 1992), uveal melanoma accounts for about 13% of all deaths from melanoma and is universally fatal within 4 to 9 months of diagnosis. High rate of mortality is due to its preferentially disseminates haematogenously to the liver with too short survival time (Eskelin *et al.*, 1999). No effective treatment of metastatic disease is yet available. Uveal melanomas arise in an immune-privileged site may be due to expressing of molecules to which the host is not tolerized (Niederkorn *et al.*, 2006; Streilein *et al.*, 2003).

NK cells area subset of bone-marrow derived lymphocytes cells that without the need for immunization or pre-activation, can recognize and kill aberrant cells, so they called natural killer (NK). They act as potent cytotoxicity agent (against not only transformed cells and tumor cells, but also infected cells with certain intracellular pathogens or viruses). In particular, the importance of NK cell comes by rapidly produce cytokines that regulate the subsequent development of specific immunity that have antimicrobial effects or prime other cells of immune system (Hamerman, *et al.*, 2005)

The expression of MHC class I in tumor cell has stimulated interest, because of its play as a major role in the interactions of aberrant cells with the host's immune cell such as cytotoxic T lymphocytes (CTLs) and NK cells. Uveal melanoma metastasis is frequently associated with abnormalities in major histocompatibility complex (MHC) class I expression. Therefore, presence of MHC class I antigens may restrict the interaction of uveal melanoma lesions as target cells with CTLs. By reducing MHC class I expression level as immune escape mechanism, tumor cellevade T cell mediate recognition and destruction, that event will makes them as a target for NK cell activities eventually (Ali, T.H., 2009).

Little is known about the role of MHC expression in progression of this malignant disease and protective role of NK cell at that time. Over the past years, it has been established that activating and inhibiting MHC class I specific receptors regulate the activity of NK cells by delicate balance between activating and inhibitory signals that inducing through receptor structures on the cell surface. These signals are triggered by and proportionate with its interaction with specific ligand on cancer cell surfaces. In general, inhibitory receptors on NK cell monitor the presence of MHC class I surface molecules as explained by the "missing self" hypothesis on tumor cell (Ka¨rre *et al.*, 1986). Eventually, it is found that loss of MHC class-I expression in primary tumors is associated with an improved survival and a lower occurrence of metastases (Blom *et al.*, 1997; Ksander & Chen,1999; Ericsson *et al.*, 2001). Therefore, MHC class I reduction on tumor cell (Missing self-phenomena) is induces of specifically NK cell antitumor activities. However, it is so appropriate for immunotherapy such as adoptive transfer of tumor-infiltrating lymphocytes (TIL) (such as NK cell) that have provided evidence at testing of the significant efficacy of immunotherapy for metastases arising from uveal melanomas (Christoph et al., 2002).

Although cutaneous melanoma has received considerable attention, few studies have focused on the role of MHC expression during progression of uveal melanoma toward the liver and still unknown the potential of NK cell based immunotherapy against uveal melanoma.

Many studies have shown that human NK cells express adhesion molecules, called CD226 (DNAX accessory molecule 1 DNAM-1). Unlike other NK cell receptors,

DNAM-1 receptor is one of NK cell activating receptor exists as an activating form, differs from most other that found in both activating and inhibitory forms. The poliovirus receptor (PVR; CD155) and nectin 2 (CD112)molecules were identified as ligands for DNAM 1 receptor express by tumors such as skin melanoma (Lakshmikanth *et al.,* 2009).

Although many years ago documented the presence of natural killer cells in TIL populations isolated from human choroidal melanomas (Ksander *et al.,* 1991), no studies up todate have examined the relative susceptibility of human uveal melanomas to NK cell-mediated cytolysis *in vitro* or *in vivo* or the capacity of NK cells to prevent the metastatic spread of uveal melanoma cells in correlation with MHC class I and activating ligands expression level.

The present study was designed to understand the NK cell recognition of uveal melanoma and study of NK behavior during their progression to liver. The evaluation of *in vitro* NK cell mediated cytotoxicity assay against allogenic primary and metastases uveal melanoma cell lines was done after analyzing of various NK receptor Iigands such as MHC-class I, PVR and nectin-2.

MATREIALS AND METHODS

Tumor cell lines: Primary uveal melanoma cell lines OCM1, OM431 (Ding *et al.,* 1995) and metastatic uveal melanoma to liver cell lines OMM1, OMM2.3 (Luyten *et al.,* 1993; Jacobus *et al.,* 2007 respectively) were maintained in complete RPMI 1640 medium. Skin malignant melanoma were established from patients and cultured as described in (Verbik *et al.,*1997, Dissanayake *et al.,* 2004) were named as 59a and 37a. Briefly tumor cells (uveal and skin melanoma cell lines) were distributed into 25-cm^2 tissue culture flasks in RPMI 1640 supplemented with 10%, heat-inactivated FCS, 2% glutamine (200mM) (Invitrogen, Milano), 100IU/ml penicillin, and 100µg/ml streptomycin in 5% CO_2 humidified atmosphere at 37°C. No feeder cell was used. The confluent cultures were passaged at 1:10 to 1:25 dilutions (Lakshmikanth, *et al*., 2009).

NK cells: Isolation of PBMC (peripheral blood mononuclear cells) from healthy donor Buffy coat was done by using Ficoll-Hypaque through density gradient centrifugation or after depletion of monocyte cells by plastic adherence. Shortly, the monocyte depletion was accomplished incubating PBMC on plastic surface for 2 hours at 37° C +5% CO_2.

Isolation of NK cells was done by using of PBMC or PBL,s negative selection (indirect magnetically separation) with cocktail biotin conjugated monoclonal antibodies. The magnetically labeled non NK are depleted by retaining them on a MACS column while the unlabeled NK cells run through the column. Each ten million PBL,s can get one million NK cells with relative purity (90%). The freshly

enriched NK cells were suspended in IMDM culture medium (Life Technology, Milan, Italy) supplemented with Penicillin (100IU/ml) and Streptomycin (100µg/ml), 10% FBS (Invitrogen). To obtain IL-2 activated polyclonal NK cells for activated NK cell cytotoxicity, 200U/ml IL-2 recombinant interleukin-2 (rIL-2) (Chiron, Emeryville, CA, USA), was added and cultured for two days, prior their usage in functional assay experiments (Lakshmikanth, *et al*., 2009).

Antibodies and immunofluorescence techniques:

for phenotypic analysis of tumor cell lines, they incubated with human serum for 15 min at room temperature then treated with primary monoclonal antibodies: W6/32HL (pan reactive anti-HLA class I mAb) B1.23.2 (anti-HLA-B, C), BB7.2 (anti HLA-A2, IgG2b) (anti-PVR, IgG1) and mAb L14 (anti-Nectin-2, IgG2a). All mAb's were used at a final concentration of 10µg/ml (Lakshmikanth, *et al*., 2009).

In the indirect staining method cells were incubated with appropriate primary mAb followed by FITC (Sigma, Italy) conjugated goat anti-mouse secondary antibody. In all experiments the isotype matched controls were used to set up the negative values, the cells were solely incubated with FITC immunoglobulin. Cells were fixed in 3% formaldehyde for data analysis. Sample fluorescence was measured by the Fluorescence-Activated Cell Sorter FACS Calibur apparatus using the Cell- Quest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Cytotoxicity assay:

to assess the cytotoxicity activity of resting and IL-2 activated NK cells against tumor targets cells *in vitro*, we cultured carboxy-fluorescein diacetate cFDA labeled target cells in constant number in 96 wells before a different effecter cell ratio to target cells (among 25/1 -1/1 E:T ratio) were added. After the incubation for 3 hours at 37° C and 5% CO_2, the results are generated by FACS caliber analysis and the percentage of target cell lysis is calculated as according this equation:

$$\%\ \text{NK based cytolysis} = \frac{\text{mean no. of tumor cells in control} - \text{mean no of tumor cells with effecter cells}}{\text{mean no. of tumor cells in control}} \times 100$$

Statistical Analysis:

Statistical computations were done using the (graph pad prism 5.0 software) for Windows. Data obtained from multiple experiments of uveal melanoma cell lines metastatic melanoma are calculated as mean ± SEM. analyzed for comparison of statistical significance by using the 2-tailed Student t-test, for independent groups. P values less than 0.05 were considered significant.

Allogeneic IL-2–activated NK cells from healthy donors were incubated at different E:T ratio in the presence of the indicated combinations of MHC masking monoclonal antibody, with tumor cell lines and subjected to cytotoxicity assays.

RESULTS AND DISCUSSION

Uveal melanoma cells expression of MHC class I and activating ligands

Major histocompatibility complex (MHC) class I antigen down-regulation is common phenomena among human tumors and is believed to be an important factor in their escape from recognition and destruction by MHC class I antigen–restricted, tumor associated CTLs (Charlotta *et al.,* 2001).In contrast, MHC class I antigen loss may result in increased sensitivity of malignant cells that invading blood vessels to NK cells (Dissanayake *et al.,* 2004). For that hematogenous route spreading tumor cell (such as uveal to liver metastasis tumor cells) will encounter tumor-NK cell based recognition and elimination. Therefore to elucidate this point, we performed differential melanoma expression analysis. The expression levels of four primary and metastases uveal to liver metastases melanomas beside two adapted skin melanoma cell lines as control and healthy lymphocytes were analyzed phenotypically. Tumor cell staining performed with specific monoclonal antibodies which either recognizes whole MHC antigens (mAb W6/32) a monomorphic determinant expressed on β_2m (beta 2 micro-globulin) associated HLA-A, -B, and -C heavy chains or measured by staining with HLA-B, C or HLA-A2 specific antibody only (Fig.1A). This staining is done at the same time with DNAM 1 ligands expression investigation (Fig.1B).

In general our data indicated that tumor cells have less MHC antigens expression that healthy lymphocyte cells (as control group) have (black color column in Fig. 1A). Collectively, healthy cells expressed significantly higher amount of HLA-BC antigens than primary uveal melanoma (PUM) and liver metastatic (LM) tumor, while PUM none expressed HLA-A2 especially (see Fig.1A). Also we found that two anatomically distinct cell line groups have a significant decreased expression of MHC class I molecule as compared to lymphatics spread melanoma (skin metastatic melanoma) (data not shown). This result ensured the down regulation of MHC-I is the predominant tumor evading of immune response for metastasis achievement.

Also our results showed that expression of activating ligands (nectine-2 and PVR) of receptor DNAM-1(CD226) was obviously higher than control cells (healthy donor's lymphocytes) in addition to nectine-2 was the higher on liver metastasis (L.M), while PVR ligands was the higher DNAM-1 ligand which expressed on primary uveal melanoma (P.U.M) (see Fig. 1B). Tumor target cells may specifically down regulate the expression of activating ligands, such as DNAM-1 upon recognition of PVR-expressing tumor cell as documented for ovarian carcinomas (El-Sherbiny *et al.,* 2007). The present study found that LN metastatic skin melanoma expressed significantly higher amount of PVR than PUM and LM.

Uveal melanomas sensitivity to NK cytolysis is inversely correlated with MHC Class I expression

Since NK cell lysis is enhanced by down-regulated MHC class I expression, Uveal melanoma may not actually represent an exception to this rule. We want to understand whether MHC class I expression level influence NK cells protective and controlling role against uveal melanoma. Therefore, we analyzed comparatively the immune-phenotype of four cell lines that represent different uveal melanoma stages in comparison with two LN cell lines. Our findings indicated that expression of high MHC class I antigen levels, make uveal tumor cells more resistant to intravenous lysis by NK cells than those with low MHC class I antigen levels (skin melanoma) (see Fig. 2 and 3).

The results indicated that NK cell-mediated cytolysis was clearly correlated with expressed class I MHC antigens as reflected by MFI values indication in Fig.1.MHC class I increase in hematologically spreaded metastasis (skin, ascites, liver) while lymph nodes expresses lower level (data not shown). IL-2 slightly increases the NK cell–mediated cytotoxicity but did not alter the preferential recognition of LN metastatic cell lines (see Fig. 2 a and b and Fig. 3)

These observations could be interpreted as a result of the elimination of tumor cells by NK cell activated by the receptors such as NCR,s and DNAM1 and inhibited by MHC-I recognizing receptors, Our results were in consistent with that previously reported (Krishnakumar *et al.,* 2003) mentioned that human leukocyte antigen (HLA) class I, β_2m and the antigen presenting machinery (APM) were decrease in 100% primary uveal melanoma and positive in 67% tumor with liver metastasis.In this respect, abnormalities in HLA class I expression have been conclusively demonstrated as one of immune evading mechanisms (Campoli *et al.,* 2002). In our experimental design MHC class I antigen expression in uveal melanoma cell lines that stained with HLA locus-specific mAbs is correlated with NK cell mediated lysis. By another way, we examined the importance of MHC reduction on the surface of tumor cell by masking the MHC-I antigen by specific mAb before encountering NK cell cytolysis. Our results revealed that masking of MHC make tumor cell more prone to attacking NK cell anti-tumor activities in comparison with non-masked tumor cells (see Fig. 3C)

This finding may facilitate understanding of fine mechanisms of receptor –ligand interaction that contribute to better design the immunotherapy approach based on the usage of NK cells like adoptive cell therapy based on NK cell clones receptor HLA mismatch.

Because of the limited number of studied cell lines, we did not perform statistical analysis to correlate DNAM 1 ligands expression and tumor cell susceptibility to NK cell immune activity, in order not to overestimate the power of our observation.

Cutaneous melanoma more NK cell sensitivity mediated lysis than uvealmelanoma.

Since loss of HLA class I antigens renders cells more sensitive to NK cell mediated lysis, due to the opposite job of NK cell with CD8 bearing T cell, NK cells may be particularly important in tumors that spread hemato-genously in comparison with their role in tumors that spread through the lymphatic system as we previously founded (Lakshmikanth *et al.*, 2009). Therefore, it has been speculated that the immune surveillance of uveal melanomas is based on NK cells (Rothenfusser *et al.*, 2002).

Our previous studies in cutaneous melanoma demonstrated changes in the inflammatory infiltrates at different tumour stages, which were associated with changes in activating ligands expression.

Expression of MHC class I in primary lesions with a favorable clinical course may reflect the susceptibility to NK-cell–mediated lysis of low HLA class I– expressing melanoma cells invading blood vessels, as proposed by (Blom *et al.*, 1997). If this interpretation is correct, NK cells may be particularly important in tumors that spread hematogenously, but may play less of a role in tumors that spread through the lymphatic system. To validate previous finding, we compared lymph node metastases cell lines (LN) sensitivity toward allogeneic NK –mediated lysis with (PUM and LM). We elucidated that as long as LN expressed less MHC-I, they was more susceptible to NK whether resting or IL-2 activated one. Also we noted that NK cell activation by IL-2 was significantly enhance its cytolytic activities against LN especially at 3:1 and 6:1 effector: target ratio (see Fig. 3c and d).

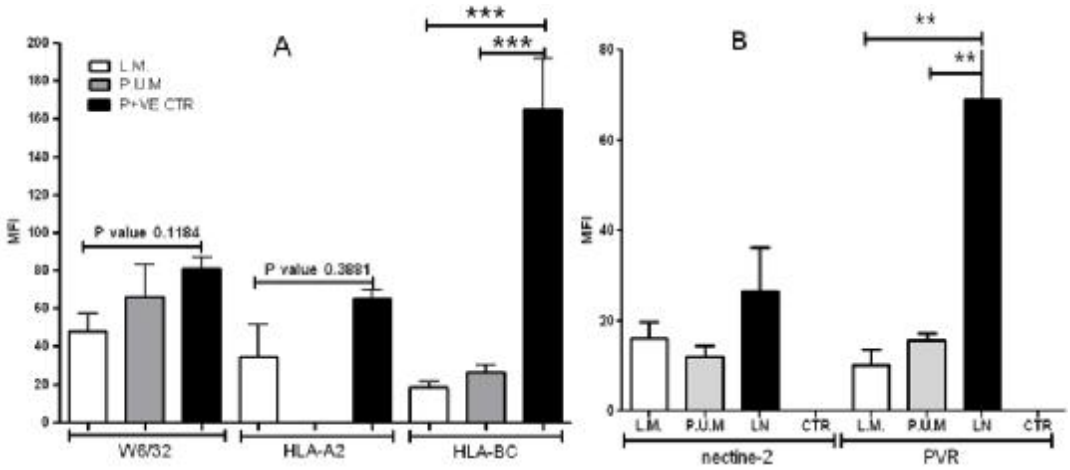

Figure 1: (A) represents the mean of MHC-1 w6/32, HLA-A2, HLA-BC, as MFI (mean fluorescent intensity) values on the surface of OMM1,OMM2.3 cell lines (liver melanoma metastasis(symbolic as LM)) OCM1, OM431 cell lines primary uveal melanoma (symbolic as P.U.M) in comparison with PBL,s as positive control

(symbolic as P+ve CTR). L.M and P.U.M expressed MHC-1 w6/32 ,HLA-BC as control healthy cells but very significant low level of HLA-BC, they doesn't express HLA-A2 so there are different susceptibility to lysis by NK cells. (B) The activating ligands of DNAM-1 receptor (nectin-2 and PVR) expression levels presented as MFI of the previous mentioned cell lines in addition to lymph node metastatic melanoma (LN).

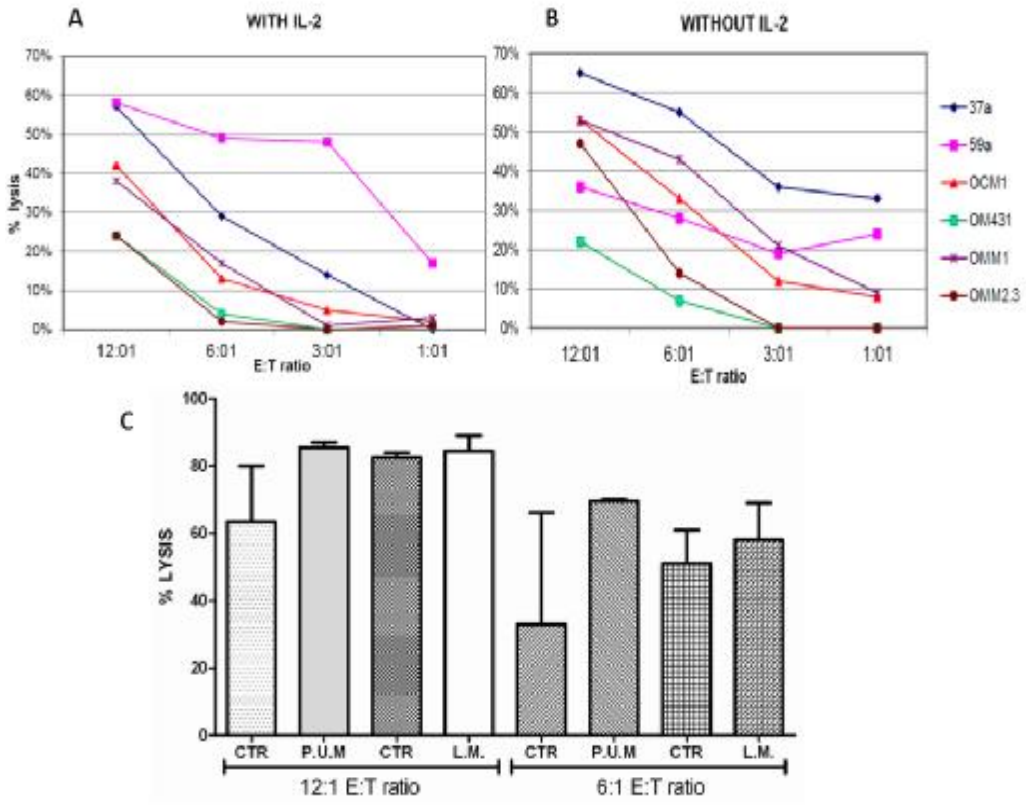

Figure 2: (A) represent results of three cytotoxicity experiments of 59a, 37a (lymph node melanoma metastasis) OMM1, 0MM2.3 (liver metastasis LM) and OM431, OCM1 (primary uveal melanoma PUM) against IL-2 activated (B) the same A setting but with resting NK cells in different E:T ratio (C) Using specific mAb for masking of MHC class I on PUM and LM tumor cells prior running of cytotoxicity against allogenic NK cells.

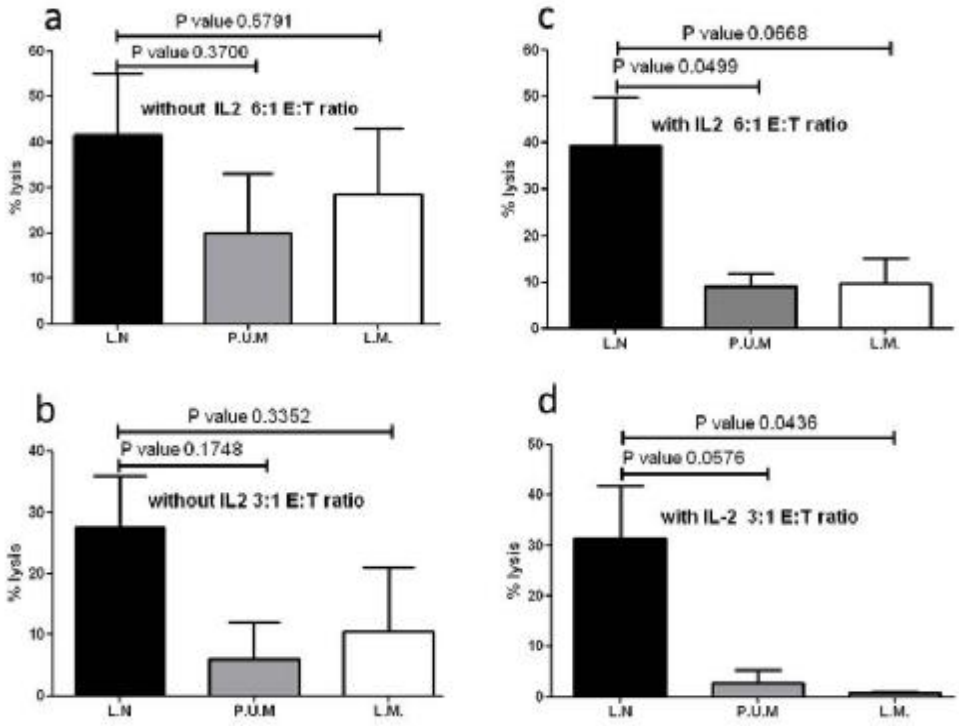

Figure 3: The results of three cytotoxicity experiments of two lymph node melanoma metastasis cell lines (symbolic as LN) two liver metastasis cell lines (LM) and two primary uveal melanoma cell lines (PUM) that put against resting (without IL-2)(a and b) and IL-2 activated NK cells(c and d) in 6:1 E:T ratio (a and c) and 3:1 E:T ratio (b and d). Note the percentages of lysis were significantly different between LN and PUM cell lines in both 3:1 and 6/1 E: T ratio, when NK cell activated by IL-2 (right two figures). There are less killing by resting NK cell as in IL-2 activated NK cell but it was not significant one (left two figures) in both studied E:T ratio mentioned above.

Acknowledgments :
The authorthank grant of the Associazione Italiana Ricerca sul Cancro (AIRC to E. carbone). Dr Soldano Ferrone, (Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, USA) and D. Cosman, (Amgen Inc. Seattle, WA, USA), for monoclonal antibodies gifts. We thank Dr, Anichini (Instituto Nazionale Tumori, Milano, Italy) for providing skin malignant melanoma cell lines

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تقييم التعبير عن معقد التوافق النسيجي من الصنف الاول وروابط مستقبل تنشيط الخلايا القاتلة الطبيعية في مزارع خلوية لميلانوما المقلة في الانسان

LijN5bx ̈ ZG

الخلاصة

تم اجراء الكشف التعبيري المظهري المناعي لعدد من السلالات الخلوية لميلانوما المقلة الابتدائي (OCM1, OM431) وللميلانوما المنبثة الى الكبد (OMM2.3, OMM1) فيما يخص معقد التوافق النسيجي من الصنف الاول وذلك عن طريق تصبيغه باستخدام الاضداد النوعية وحيدة النسيلة الخاصة بمستضدات HLA-A2, HLA-BC وكذلك الضد العام لجزيئة HLA-class I . كذلك جرى التحري عن التعبير المظهري المقارن لروابط مستقبل التنشيط DNAM-1 وهي (PVR وnectin-2) خلال الانبثاث الى الكبد وربطت النتائج بمستوى حساسية الخلايا الورمية للقتل بواسطة الخلايا القاتلة الطبيعية. وقد قدرت النتائج باستخدام العدّ الخلوي الجرياني flow cytometry. كل المزارع الخلوية التي جرى التحري فيها قد عبرت عن مستوى منخفض من معقد التوافق النسيجي من الصنف الاول عند مقارنتها مع خلايا الدم المحيطي السليمة والذي كان معنوياً في حالة HLA- BC ،حيث اظهر الارتباط الوثيق بميلانوما مقلة العين uveal melanoma . بينما عبرت الخلايا الورمية عن روابط مستقبل التنشيط للخلايا القاتلة الطبيعية DNAM1 المرتبطة بزيادة حساسيتها للقتل بواسطة الخلايا NK لنفس المستوى مما في خلايا ميلانوما الجلد. هذه النتائج جاءت متوافقة مع الدور المعلوم للخلايا القاتلة الطبيعية في السيطرة على الخلايا الورمية المنبثة عن طريق المجرى الدموي والتي ظهر ان لها دوراً اقل مما في الجهاز اللمفاوي.اظهرت نتائج الدراسة بان التعبير عن روابط تنشيط الخلايا القاتلة الطبيعية DNAM1مرتبط مع تقدم مرحلة المرض في ميلانوما مقلة العين إضافة الى الانخفاض المتوقع في مستوى التعبير عن معقد التوافق النسيجي الذي كان مترافقاً مع تقدم المرض.