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SUSCEPTIBILITY OF HUMAN WM MELANOMA CELL LINES TO NK AND LAK CYTOTOXICITY AND THEIR RELEVANCE TO THE LEVEL OF MHC CLASS I AND ICAM-1 ANTIGEN EXPRESSION

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ABSTRACT

The effect of natural killer (NK) cells and lymphokine activated killer (LAK) cells was studied on a group of human melanoma cell lines. Peripheral blood from healthy volunteers was utilized as a fresh source of natural killer cells and rhIL-2 for producing LAK cells. The cytotoxicity of effector cells was quantified using a 4 hour ^{51} determining the density of antigen expression on tumor cell surface. The majority of WM melanoma cell lines were relatively resistant to lysis by NK cells, but all showed high sensitivity to LAK cytotoxicity. The expression of MHC class I and ICAM-1 on tumor cell lines varied, however using linear regression analysis between MHC class I and ICAM-1 antigen expression, and UK/
pression of these antigens on the WM melanoma cell lines tested are independent of NK/
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INTRODUCTION

Natural killer (NK) cells are bone marrow derived and thymus independent, and are operationally defined by their ability to lyse a restricted panel of target cells without prior "antigen specific priming".[1] NK cells have been considered to be effector cells that lyse tumor cells or virally infected cells via non-specific mechanisms.[2] NK activity *in vitro* is strongly stimulated by pre-treatment of effector cells with IL-2 or IFNs, after which they become lymphokine activated killing cells or LAK, and do not undergo cell division.[3,4]

Several tumor cell lines of different origin as well as normal cells including hepatocytes, peripheral blood leukocytes (PBL), bone marrow cells and thymocytes are essentially resistant to NK/LAK activity.[5]

In "adoptive immunotherapy", LAK cells are injected I.V. into tumor-bearing animals or cancer patients.[6] However, *in vivo* studies showed only a 25-30% response to administration of LAK cells and/or IL-2 among different tumors.[7] It whould therefore seem that the mechanisms involved need to be further studied. The data which describes the mechanisms of susceptibility or lack of susceptibility to NK/LAK is controversial.[8,9] In addition, several different mechanisms, including the role of antigen expression on the cells, have been considered.[7,10,11] So, an investigation into the relationship of surface molecules on target cells and NK/LAK cytotoxicity might be important if we are to develop a better understanding of the mechanisms of lysis.

We present the results of our investigations on the role of class I and adhesion molecule receptors on target cells from a group of paired (primary and metastatic) melanoma

cell lines, in order to determine their susceptibility to NK/LAK activity *in vitro*. The ability of NK/LAK cells to recognise and destroy target cells by multiple adhesion-like receptors on target cells[11] was included. Intracellular adhesion molecule-1 (ICAM-1) and major histocompatibility complex (MHC) class I have been classified as the members of the immunoglobulin superfamily and are involved in immune function and cell killing.[10,12] Therefore, the susceptibility of the tumor cell lines to NK/LAK cytotoxicity, and the relationship between the constitutive level of expression of these proteins (MHC class I and ICAM-1 antigen) and effector cytotoxicity was investigated.

MATERIALS AND METHODS

This study was based on a group of human melanoma cell lines derived from primary (P) and metastatic (M) tumors[13] as follows: (primary/metastatic); WM278/WM1617; WM1361A/WM1361C; WM793/WM1205; WM983A/WM983B; and WM35(P); WM902B(P); WM39(P). Lymphoblastoid tumor cells (K562, NK sensitive) and colon carcinoma cells (SW742, NK insensitive) were used as controls. A375, a cutaneous melanoma cell line, was included. Melanoma cell lines were cultured in Dulbecco's medium (Life Technologies Ltd; Paisly, Scotland) and peripheral blood mononuclear cells (PBMC), K562 and SW742 cells in RPMI (Life Technologies Ltd; Paisly, Scotland) plus 10% fetal calf serum (Northumbria Biologicals Ltd., UK) at 37°C in a 5% CO_2 humidified incubator.

The following antibodies were used;

1) IgG_2 mouse monoclonal antibody W6/32 against human HLA class-I antigen (a generous gift from Dr. K. Gelsthorpe, BTS, Sheffield).

2) Anti-ICAM-1, CD54 against the intracellular adhesion molecule (a generous gift from Dr. N. Hogg, ICRF, London).

3) FITC-conjugated goat anti-mouse (Fab)2 fragment immunoglobulin) second stage antibody alone was used as the negative control for the test.

Tumor cells were prepared in the following way:

5-10×10^5 tumor cells were seeded into tissue culture flasks and incubated in media containing 10% FCS. Cells were then harvested as follows: media was removed and the cells washed twice using PBS. 2mL versine (0.2 g EDTA in 1mL PBS) was added, incubated at 37°C for 5-10 minutes or until cells become detached, and the cells removed and washed/centrifuged in PBS at 1900 r.p.m. for 5 minutes at 4°C. The supernatant was discarded and cells aliquoted into 1×10^5 cells/test tube and labelled with the primary antibody (10μL), incubated for 20-30 minutes at 4°C, with occasional mixing. Cell samples were then washed with PBS/serum at 4°C. The second-stage antibody (FITC) was then added at 100μL (1 in 100 dilution) and incubated at 4°C in the dark for 30 minutes, with occasional mixing. Samples were washed once more and the pellet re-suspended in 300μL PBS/serum. Samples were then ready for flow cytometric analysis.

For flow cytometric Ab analysis, cell suspensions were run on an Orthocyte benchtop flow cytometer (Ortho Diagnostics Ltd.) with measurements taken of the median fluorescence channel, from a total of 10,000 cells per sample. The median fluorescent channel for each fluorescence histogram was considered to represent the average amount of antibody bound to each cell, and therefore be used as an indicator of antigen density for the protein under analysis. A representative flow-cytometry printout which indicates the intensity of antigen expression from the tested cells is illustrated in Figure 1.

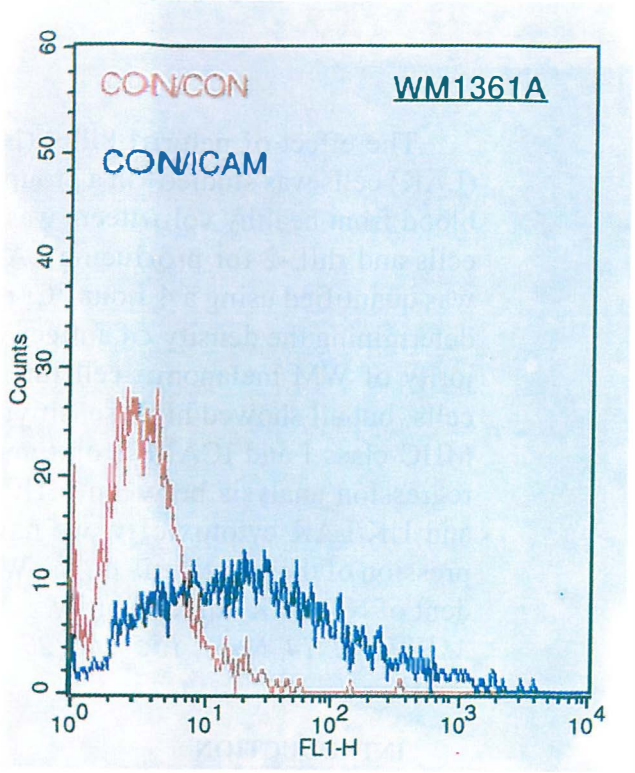

Fig. 1. The flow cytometric analysis of stained cells with MoAb against ICAM-1 as well as the negative (control) outlined is shown as a fluorescence histogram and represent the average level of antibody binding and antigen expression (fluorescence intensity) on WM1361A cells.

Cytotoxicity assay

Peripheral blood was obtained from normal healthy volunteers. Heparinized blood was layered over Histopaque 1077 (density= 1.077g/m-Sigma) and centrifuged for 35 minutes at 400 g. The buffy coat interface was collected and washed twice with cold PBS. The resultant mononuclear cell preparation was utilized as a fresh (PBMC) source of natural killer (NK) cells, and PBMC cultured in the presence of recombinant rhIL-2 (500μ/mL) in 24-48 hours for producing LAK cells. Lysis of tumor cells by NK/LAK cells

was quantified using a 4 hour ^{51}Cr release assay. Tumor cells were labelled with 100mCi ^{51}Cr for an hour, washed three times with RPMI and the pellet was aliquoted at a density of 1×10^4 cells per well on 96-well round bottom plates. NK/LAK cells were then added to the wells to achieve effector to target (E:T) ratios of 50:1, 25:1, 12:1 and 6:1. Triplicated wells were set up for each E: T ratio. After a 4-hour incubation at 37°C, the supernatant was harvested (Skatron system) and counted. Spontaneous release was measured from 1×10^4 cells to which media alone was added, and the maximum release was measured by lysing the same number of target cells with 1% sodium dodecyl-sulphate. Spontaneous release remained below 15% for all experiments. The percent specific lysis was calculated using the following formula:

$$\%\text{lysis} = \frac{(\text{experimental CPM} - \text{spontaneously released CPM})}{(\text{maximum CPM} - \text{spontaneously released CPM})} \times 100$$

Statistical analysis was performed using the Student's t-test for antigen expression and linear regression for correlation of NK/LAK and antigen expression.

RESULTS

The results of the cytotoxicity assays showed that the majority of WM melanoma cell lines were relatively resistant to lysis by freshly isolated PBMC (NK). Although NK cytotoxicity varied from target to target, cytotoxicity less than 8% for a ratio of 50: 1 is considered to indicate resistance, which is similar to the data generated by the insensitive (SW742) cell line. In addition, A375 and WM35 were weakly sensitive to NK cytotoxicity in comparison with the control cells, e.g. K562 with 39.6% cytotoxicity at a ratio of 50:1 (Table I). When the PBMC were treated with IL-2 for 24-48 hours, LAK cells were produced and the cytotoxicity assays showed that all cell lines tested were sensitive to LAK cytotoxicity. The percentage of LAK cytotoxicity ranged from 44.4 to 73 for the 50:1 ratio and 19.11 to 54.6 for 12:1, as indicated in Table I. The tumor cell lines previously shown to be either sensitive or resistant to NK killing became highly

Table I. Basal levels of ICAM-1 and MHC class I expression as well as the susceptibility of the tumor cell lines to NK and LAK cytotoxicity.

Constitutive expression of			%Cytotoxicity; E: T ratio			
			LAK	LAK	NK	NK
Cell lines	ICAM-1	MHC Class I	50:1	12:1	50:1	12:1
K562	60±7	40±6	83	62.2	39.6	26:7
SW742	81±8	166±11	76	47.2	3.7	0.6
WM793(P)	303±15	195±18	57.9	33.5	4.6	2.09
WM1205(M)	354±98	211±56	49.7	22.9	3.4	1.99
WM278(P)	184±31	378±97	64	40.3	6.8	0.79
WM1617(M)	422±96	443±101	69.8	38.5	7.49	2.9
WM983A(P)	155±12	599±24	48.1	22.8	0.42	0.45
WM983B(M)	180±36	337±35	44.4	27.5	2	0.28
WM1361A(P)	63±22	292±85	53	27	2.7	1.5
WM1361C(M)	137±63	150±10	51	19.11	6.7	2.7
A375	120±6	189±23	73	53.5	13.2	8.9
WM35(P)	252±32	260±21	69.4	54.6	11.4	7.6
WM902B(P)	315±27	430±32	49.3	20.2	3.6	0.2
WM39(P)	282±24	355±33	66.1	37.5	5.6	0.3
r^2 for ICAM-1			0.033	0.000	0.000	0.025
(P value) for ICAM-1			(0.571)	(0.965)	(0.981)	(0.618)
r^2 for Class I			0.024	0.040	0.215	0.212
(P value)			(0.623)	(0.531)	(0.128)	(0.131)

The data presented for MHC class I and ICAM-1 is mean±S.E. of 3-5 experiments measuring the median channel of fluorescence intensity. r^2= regression coefficient, P value for the data presented, using SPSS software. The data presented for NK and LAK cytotoxicity, obtained by using the same source of PBMC effector cells and measured in 4 hours ^{51}Cr release assay. (P)= Primary cell line, (M)= Metastatic cell line.

sensitive to LAK cytotoxicity *in vitro* (representative graph from WM793 line is illustrated in Figure 1). The quantity (mean±S.E.) of the antigen expression was obtained from 3-5 separate experiments. Antigen expression was seen to vary among the cell lines (Table I). All the cell lines were found to constitutively express both proteins, as shown by the intensity (median fluorescence channel number) of ICAM-I and MHC class I antigen expression on the tumor targets. As shown in Table I, MHC class I and ICAM-1 antigen expression ranged from 150 to 599 (mean fluorescent channel number) for class I and 63 to 422 for ICAM-I (control cell lines are excluded). A representative flow-cytometry histogram is illustrated in Figure 1 to show the quantity of antigen expression for WM1316A cells labelled with anti-ICAM-1.

Representative data for cytotoxicity is shown graphically for both NK and LAK cytotoxicity for ratios of 6:1, 12:1, 25:1 and 50:1 (Figure 2). Using linear regresion analysis, there was no statistically significant correlation between NK cytolysis and MHC class I expression for all melanoma cell lines (r^2=0.215, p= 0.128, for ratios of 50:1 and r^2= 0.212, p= 0.131 for ratios of 12:1 respectively), or ICAM-1 antigen expression at any ratio (r^2= 0.000, p= 0.981, for the 50:1 ratio and r^2= 0.025, p= 0.618 for the 12:1 ratio respectively). The efficiency of killing at a ratio of 50:1 and 12:1 only is included in Table I. The data for ratios of 25:1 and 6:1 is not shown.

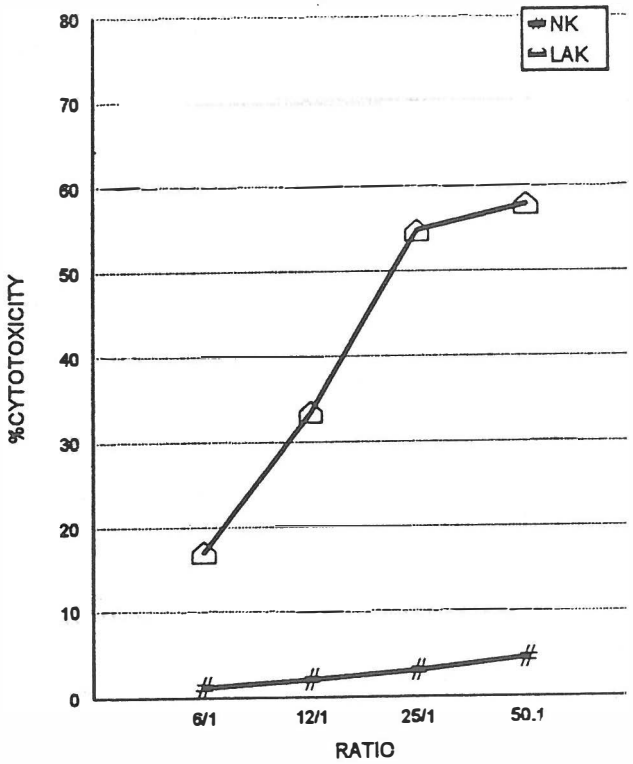

Fig. 2. Representative data for WM793 cells in terms of susceptibility to NK and LAK cytotoxicity,
tor target cell populations.

Cell lines with low ICAM-I expression, such as K562, SW742 and WM1361C, showed a similar level of LAK susceptibility to cell lines with a higher ICAM-1 expression, e.g. WM1617, WM793. Linear regression analysis did not show any significant correlation between the expression of ICAM-I and susceptibility to LAK cytotoxicity (r^2= 0.033, p= 0.571, for the 50:1 ratio and r^2= 0.000, p= 0.965 for the 12:1 ratio respectively) (Table I). Similar results were obtained for the relationship between LAK cytotoxicity and the level of MHC class I antigen expression (r^2= 0.024, p= 0.623, for the 50:1 ratio and r^2= 0.040, p= 0.531 for the 12:1 ratio respectively). Analysis of all the data obtained from these experiments shows a p value higher than 0.05.

Table II indicates the percentage of LAK cytotoxicity with different individuals (i.e., different source of effectors) against two representative tumor cell lines (Data from the other cell lines is not shown). the ability of cytotoxic killing of effector cells varied between the donors, but the ranges of lack of NK sensitivity (less than 8% cytotoxicity, data not shown) and LAK sensitivity were constant.

Table II. Percent cytotoxicity and LAK of different individuals against two melanoma cell lines (representing target cells).

	%Cytotoxicity; LAK/target ratios			
Target cells	**Effector**	**50/1**	**25/1**	**12/1**
WM278	1	83.6	66.	49.3
WM278	2	79.	88.	55
WM278	3	90.9	92.4	55.
WM278	4	64	74.6	46.
WM1617	1	52.	40.9	24.2
WM1617	2	73.	69.	48.
WM1617	3	84.	81.7	47.4
WM1617	4	69.	75.	38.

Donor variations can be seen between the 4 experimental data sets shown.
also seen between the tumor cell lines. As a result of this biological variation, individual donors were used as a source of effector cells,
cells is comparable as shown in Table I.

DISCUSSION

Studies have supported a role for NK cells in controlling the metastatic spread of tumor cells in a variety of animal models,[14] although the mechanisms involved need to be identified. Cytotoxicity mediated by NK cells shows considerable variability in sensitivity between different cell lines.[15,16] When evaluating the sensitivity of target cells to lysis, several factors play an important role in determining susceptibility to killing. For example, the ability of a cell line to bind to NK cells is one such requirement which de-

pends on the expression of cell surface receptors and antigens, and the integrity of the cell membrane.[17] Although NK cell lysis is often defined as being specific for tumor and virus infected targets, it is often difficult to demonstrate lysis of malignant cells unless NK cells are activated.[4]

The results given here show that A375 and WM35, as well as K562 were sensitive to NK cytolysis (K562 was 3.5 times more sensitive than the other lines). However, all other primary and metastatic WM melanoma cell lines tested were resistant to NK cell killing at the 50:1 ratio (less than 8%). In agreement with data achieved in this study, Ma et al.[18] demonstrated that the susceptibility of human uveal melanoma cell lines to NK cell mediated cytolysis was also variable.

The results of this investigation have demonstrated a variation in the efficiency of LAK killing using different individual PBMC populations against the same tumor cell line, or the same PBMC population against different targets. In addition, all cell lines tested, including both NK resistant and NK sensitive, showed a high degree of susceptibility to lymphokine activated killer (LAK) cells generated by rhIL-2. In contrast with this data, several tumor cell lines have been demonstrated which were resistant to LAK susceptibility.[8]

Structures recognized on the target cells that confer sensitivity to LAK cell killing have also been elusive.[19] The failure of fresh, unactivated NK cells to lyse most tumor target cells may primarily reflect the lack of expression (or expression in low density) of the appropriate activation receptors. The ability of rhIL-2 activated NK cells (i.e., LAK cells) to efficiently lyse a wider spectrum of tumor cells may reflect the degree of expression of appropriate receptors on the effector cells as a consequence of their activation status.[2,20]

Many tumor cells susceptible to lysis mediated by NK/LAK cells express moderate to high levels of class I antigens. Thus either MHC class I antigens do not function as specific protective elements for allogeneic NK cell cytolysis or possibly the tumor cells may lack the expression of single class I alleles capable of functioning as a protective element.[2,20] The data given here (which was generated both by using a single donor for generating NK and LAK cells, as well as several donors) showed no correlation between class I expression on melanoma cells and susceptibility to LAK cytotoxicity and supports the view of some researchers[8,21,22] who demonstrated that LAK/NK killing was independent of MHC class I antigen expression. However, Storkus et al.[9] demonstrated that transfection of polymorphic class I negative tumor cell lines had a protective effect on NK/LAK lysis,whilst transfection of other alleles e.g. HLA-A2 did not. This suggests that the conformational structure of different polymorphic class I antigens may influence the efficiency of blocking of NK/LAK killing.

A difference in sensitivity to NK lysis between primary and metastatic cells has been reported[6] and it has been suggested that the metastatic cell represents the main NK-target cells *in vivo*.[23,24] However, this hypothesis is not in agreement with our results, which showed no significant difference between primary and metastatic melanoma cell lines to NK-mediated cell cytotoxicity.

Other studies also failed to demonstrate an absolute correlation between MHC class I expression and NK sensitivity in cell lines.[8,21,25] Moreover, NK susceptibility was found to be independent of HLA class I expression on a comprehensive panel of astrocytoma, meningioma, lung, breast and colon carcinoma cell lines.[21,26]

Chong et al.[27] demonstrated that cell adhesion molecules can provide signalling to induce cytotoxic activity in NK cells. Our data showed that all cell lines used in this study were positive for ICAM-1, and whilst the constitutive level of expression varied, all were insensitive to NK cytotoxicity. This may be the reason behind one reported observation that ICAM-1 expression in uveal melanoma was correlated to tumor size. Larger tumors (generally more aggressive and metastatic) expressed more ICAM-1.[28] Other studies have shown that ICAM-1/ICAM-2 dependent interactions may have significant roles in NK recognition at least for some target cells.[29]

However, the data obtained in this study showed no correlation between the level of ICAM-1 expression and NK/LAK susceptibility. Therefore this study supports the view of Kondo who stated that molecules other than ICAM-1 or HLA class I may be responsible for target susceptibility to lysis by NK/LAK cells.[30] It is suggested that tumor cells which establish metastases are intrinsically more NK/LAK resistant,[5] yet our findings did not show a constant or significant difference in the susceptibility to LAK cytotoxicity between the primary and metastatic lines. In addition, whilst the cytotoxic potential of effector cells is different among individuals, the ranges of insensitivity to NK and sensitivity to LAK remained constant for all the tumor cell targets.

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REFERENCES

1. Ortaldo JR,
cells:
ness.
2. Moretta L, Ciccone E, Mingari MC,
Human natural killer cells:
receptors,
3.
Rev 6: 637-651, 1987.
4. Reiter ZVI,
lation of MHC-unrestricted cell mediated cytotoxicity.

cal Reviews in Immunology 13(1): 1-34, 1993.
5. Hiserot JC: Lymphokine activated killer cells: biology and relevance to disease. Cancer Invest 11: 420-439, 1993.
6. Reiter Z: Interferon-a major regulator of natural killer cell-mediated cytotoxicity. J Interferon Res 13: 247-257, 1993.
7. WE, (ed.), Immunology Recognition and Response. New York: W.H. Freeman and Company, pp. 109-120, 1991.
8. Nouri AM, Hussain RF, Dos-Santos AV, et al: Intensity of class I antigen expression on human tumor cell lines and its relevance to the efficiency of non-MHC-restricted killing. Br J Cancer 67: 1223-1228, 1993.
9. Storkus WJ, Alexande J, Payne JA, tance to natural killing; identification of nonpermissive residues in HLA-A2. Proc Natl Acad Sci USA 88: 5989-5992, 1991.
10. Raulet DH, Werner H: Natural killer cell receptor: the offs and ons of NK cell recognition. Cell 82: 697-700, 1995.
11. Trinchieri G: Recognition of major histocompatibility complex class I antigens by natural killer cells. J Exp Med 180: 417-421, 1994.
12. Siu G, Hedrick SM, Brian AA: Isolation of the murine intercellular adhesion molecule-1 (ICAM-1) enhances antigen-specific T cell activation. J Immunol 143: 3813-3820, 1989.
13. Herlyn M: Human melanoma: development and progression. Cancer Met Rev 9: 101-112, 1990.
14. Shpitz B, Fernandes BJ, Mullen JB, et al: Improved engraftment of human tumours in SCID mice pre-treated with radiation and anti-asialo GM1. Anticancer Res 14: 1927-1934, 1994.
15. Henkart PA, Lewis JT, Ortaldo JR: Preparation of target antigens specifically recognised by human natural killer cells. Natural Immunity and Cell Growth Regulation 5: 113-126, 1986.
16. Laskay T, Kiessling R: Interferon and butyrate treatment leads to a decreased sensitivity of natural killer target cells to lysis by homologous but not by heterologous effector cells. Nat Immunity Cell Growth Regul 5: 211-220, 1986.
17. Timonen T Patrarroyo M, Gahmberg CG: CD11a-c/CD18 and GP84(LBB-2) adhesion molecules on human large granular lymphocytes and their participation in natural killing. J Immunol 141: 1041-1046, 1988.
18. Ma D, Luyten GP, Luider TM, et al: Relationship between natural killer cell susceptibility and metastasis of human uveal melanoma cells in a murine model. Invest Ophthalmol Vis Sci 36: 435-441, 1995.
19. DeFries RU, Golub SH: Interferon-gamma reduces the sensitivity of cultured and fresh human tumor cells to lysis by lymphokine activated killer cells. Nat Immun Cell Growth Regul 7: 65-76, 1988.
20. Ferrini S, Miescher S, Zocchi MR, et al: Phenotypic and functional characterisation of recombinant interleukin-2 (rIL-2) induced activated killer cells. Analysis at the population and clonal levels. J Immunol 138: 1297-1305, 1987. Rosenberg
21. Pena J, Solana R, Alonso MC, et al: MHC class I expression on human tumor cells and their susceptibility to NK lysis. J Immunogenetics 16: 407-412, 1989.
22. Uchida A, Kariya Y, Inoue N, et al: Biological significance of autologous tumour killing, In: Goldfarb RH, Whiteside TL, (eds.), Tumor Immunology and Cancer Therapy. New York: Marcel-Dekker, pp. 195-206, 1994.
23. Ljunggren HG, Karre K: Variations in MHC antigen expression in tumour and its significance. Experimental strategies and interpretations in the analysis of changes in MHC gene expression during tumour progression. Opposing influences of T cells and natural killer mediated resistance? J Immunogenet 13: 278-287, 1986.
24. Lopez-Nevot MA, Esteban F, Ferron A, et al: HLA class I expression on human primary tumour and autologous metastases: demonstration of selective losses of HLA antigens on colorectal, gastric and laryngeal carcinoma. Br J Cancer 59: 221-226, 1989.
25. Aosi FC, Ohlan C, Ljunggren HG, et al: Different types of allospecific CTL clones identified by their ability to recognise peptide loading defective target cells. Eur J Immunol 21: 2767-2771, 1991.
26. Moller P, Hammerling GJ: The role of surface HLA-A, B, and C molecules in tumour immunity. Cancer Surveys 13: 101-127, 1992.
27. Chong AS, Boussy IA, Jiang XL, et al: CD54/ICAM-1 is a costimulator of NK cell-mediated cytotoxicity. Cell Immunol 157: 92-105, 1994.
28. Lawry J, Currie Z, Smith MO, et al: The correlation between cell surface markers and clinical features in choroidal malignant melanomas. Eye 1999 (in press).
29. Storkus WJ, Dawson JR: Target structures involved in natural killing (NK): characteristics, distribution, and candidate molecules. Crit Rev Immunol 10: 393-416, 1991.
30. Kondo S, Miyatake S, Kikuchi H, et al: Mechanism of interferon gamma-induced protection of human gliosarcoma cells from lymphokine activated killer lysis; division of lymphokine activated killer cells into natural killer and T-like cells. Neurosurgery 31: 534-540, 1992.