Human osteosarcoma cell susceptibility to natural killer cell lysis depends on CD54 and increases after TNFα incubation

Erminia Mariani[a,b,*], Andrea Tarozzi[a], Alessandra Meneghetti[a], Luca Cattini[a], Andrea Facchini[a,b]

[a] *Laboratorio di Immunologia e Genetica, Istituto di Ricerca Codivilla-Putti, I.O.R., Via di Barbiano 1/10, 40139 Bologna, Italy*
[b] *Dipartimento di Medicina Interna e Gastroenterologia, Università degli Studi di Bologna, Bologna, Italy*

Received 20 December 1996; revised version received 17 February 1997

Abstract Osteosarcoma cell lines vary widely in their susceptibility to natural killer (NK) cell lysis in vitro although it is still unclear why this occurs. In this study we investigated the expression of some cell adhesion molecules on osteosarcomas to determine which of these can modify the susceptibility to NK lysis and we also attempted to modulate the cytolytic susceptibility of these targets with TNFα. We found that osteosarcoma lysis induced by NK cells correlates with different expression of the CD54 adhesion molecule on osteosarcomas and the increased susceptibility after TNFα treatment mostly depends on the expression of CD54 molecules on target cells.

Key words: Osteosarcoma cell line; NK cell lysis; CD54; Tumor necrosis factor

1. Introduction

Natural killer (NK) cells are one of the lytic effector cells present among human peripheral blood mononuclear populations, involved in the defence mechanism against tumor and virally infected cells. These elements are CD16, CD56 positive, but lack CD3 which differentiates them from T cells and makes them capable of spontaneous target lysis [1].

Different studies demonstrated that susceptibility to NK cell lysis is inversely influenced by the level of HLA class I antigen on target cells [2–6] and also by intercellular adhesion molecules which play a role in the interaction between target and effector cells [7]. Cell adhesion to the target is essential in establishing leukocyte-mediated cytolysis so that lytic mediators secreted by the NK cells can maintain high concentrations at the site of junction with the target [8].

Osteosarcomas are bone-derived malignant tumors with vigorously invading capacity. They are characterized by an active proliferation of undifferentiated, atypic and often mitotic osteoblastic cells [9–12]. Despite advances in surgical technique and the development of aggressive adjuvant chemotherapy, pulmonary metastasis is still the major cause of death in patients with osteosarcoma.

To investigate the role of NK cells in the control of osteosarcoma proliferation we evaluated the susceptibility of different osteosarcoma cell lines to NK cytolytic activity in vitro. A considerable variability in susceptibility to lysis was observed and it is currently unclear why this occurs.

In this study we investigated whether a different expression of some cell adhesion molecules and their ligands on osteosarcoma cells could determine a different susceptibility to NK lysis. In addition, we tested the effect of treatment with TNFα (a cytokine that plays an important role in pathophysiology of bone remodelling [13]) of osteosarcoma cells on susceptibility to NK cell lysis and also attempted to modulate the susceptibility of target cells.

We demonstrated that TNFα-treated osteosarcoma cells have an enhanced susceptibility to NK cell lysis which mostly depends on the increased expression of CD54 molecules on target cells.

2. Materials and methods

2.1. Effector cells

Effector lymphocytes were separated from the peripheral blood of 12 healthy human volunteers (mean age ± S.D. 30 ± 2 years) by density gradient centrifugation and depleted of monocytes by absorption to plastic. Lymphocytes were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 4 mM glutamine, gentamicin (200 μg/ml) and used in cytotoxicity testing.

2.2. Target tumor cells

HOS, Saos-2, MG-63, and U-2 OS human cell lines are bone tumors with an undifferentiated osteoblastic-like phenotype [9–12]. All the cell lines were obtained from ATCC (Rockville, MD, USA). They grow as adherent cells and were routinely passaged in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 4 mM glutamine and gentamicin (200 μg/ml). Before use in the experiments, cells were detached from monolayers by brief exposure to a solution of trypsin-EDTA (Sigma, USA), washed twice and resuspended in the same medium.

2.3. Immunofluorescence analysis

We verified the expression on osteosarcoma lines of a large panel of human antigens: CD11a, b, c, CD18, LFA-2 (CD2), LFA-3 (CD58), ICAM-1 (CD54), ICAM-2(CD102), ICAM-3 (CD50), N-CAM (CD56), VLA 1–6 (CD49a–f) and CD29.

CD11c, CD54, CD49b, c, f (Cymbus Bioscience Ltd., UK), CD2, CD56 (Becton Dickinson, USA), CD29 (Endogen, USA), CD49a (T Cell Diagnostic, USA), and CD49e (Immunotech, France) were commercially available FITC- or PE-conjugated monoclonal antibodies (MoAb). CD11a (LFA-1α, TS1/22 clone), CD11b (LM2/1 clone), CD18 (LFA-1β, TS1/18 clone), CD58 (TS2/9 clone), CD102 (KS128 clone), and CD49d (KD4-13 clone) were obtained from culture supernatants of hybridomas.

Incubation of 2×10^5 osteosarcoma cells with MoAb was performed for 30 min at 4°C, followed by the secondary antibody (goat anti-mouse FITC immunoglobulin, 1:20 dilution, Becton Dickinson, USA) when the MoAb was not directly conjugated with fluorochrome. Incubation with 1:10 mouse normal serum (Dako, Denmark) was performed to reduce non-specific binding. Negative control cells were only incubated with Ig isotype control and/or with FITC-conjugated goat anti-mouse Ig.

Analysis was performed with flow cytometry using a FACStar plus cell-sorter (Becton Dickinson, USA) calibrated for fluorescence inten-

*Corresponding author. Fax: (39) (51) 6366807.
E-mail: marianie@alma.unibo.it

PII S0014-5793(97)00247-0

sity measurements using FITC or PE microbead standards (Quantum 26, Flow Cytometry Standard Corporation, PR) with assigned MESF values (molecules of equivalent soluble fluorochrome) [14–16].

2.4. Cytokine treatment

Osteosarcoma cells (2×10^6) were incubated with rHu TNFα (50 ng/ml; Boehringer Mannheim, Germany) for 24 h; untreated cultures were used as controls. After incubation, osteosarcoma cells were detached with trypsin-EDTA (Sigma, USA), washed twice and adjusted to the appropriate cell concentration using culture medium. Cell viability was determined with the eosin dye exclusion test.

2.5. Cytotoxicity assay

After incubation with or without cytokines for 24 h, osteosarcoma cells (2×10^6) were labelled with 100 μCi of ^{51}Cr (specific activity 400–1200 Ci/g) (NEN, Germany) for 1 h at 37°C with occasional shaking. Tumor cells were then washed three times with cold medium, centrifuged and resuspended at a concentration of 10^4/ml.

Varying numbers of effector lymphocytes (from 5×10^5 to 5×10^3 in 100 μl) were added to 50 μl of target cells (E/T ratios from 100/1 to 1/1). Cells were seeded in triplicate in 96-well V-bottomed plates (Nunc, Denmark), incubated for 4 h at 37°C and centrifuged.

Supernatant was harvested from each well using a collection system containing cellulose acetate absorption cartridges (Skatron, Norway) and counted in a gamma counter.

The percentage of ^{51}Cr release was calculated as: (experimental release−spontaneous release)/(maximum release−spontaneous release)×100. Spontaneous release represents the radioactivity of the target cells alone and maximum release the radioactivity of target cells lysed with 1% Triton-X100. Spontaneous release was as high as 15% of maximum release [17].

2.6. Blocking assays

For the blocking experiments, target cells (1×10^6/ml) were incubated with different concentrations of CD54 MoAb (4 μg/ml; 0.02 μg/ml; 0.008 μg/ml) for 30 min, immediately following incubation with ^{51}Cr, while effector lymphocytes were incubated with CD11a/CD18 MoAb (4 μg/ml) for 30 min, immediately before seeding.

The cells were incubated in purified MoAb or hybridoma supernatant and in medium alone. After incubation the cells were washed twice and used as targets and effector, respectively, for cytotoxicity assay.

2.7. Statistical analysis

The experimental data are expressed as means ± S.E.M. Statistical significance was tested using Student's *t*-test for paired data. The Statistics for Windows package was used to perform statistical analyses.

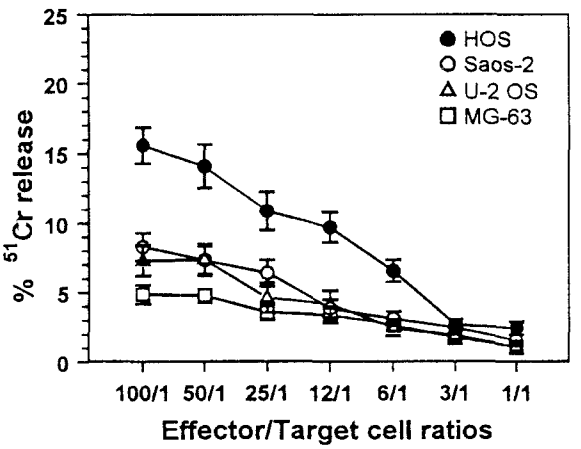

Fig. 1. Natural killer cell cytolytic activity against human osteosarcoma cell lines. Results are expressed as mean percentages ± S.E.M. Significant differences between HOS and Saos-2, U-2 OS, MG-63 cells for effector/target cell ratios: 100/1 and 12/1, $P<0.0002$; 50/1 and 6/1, $P<0.001$; 25/1, $P<0.02$.

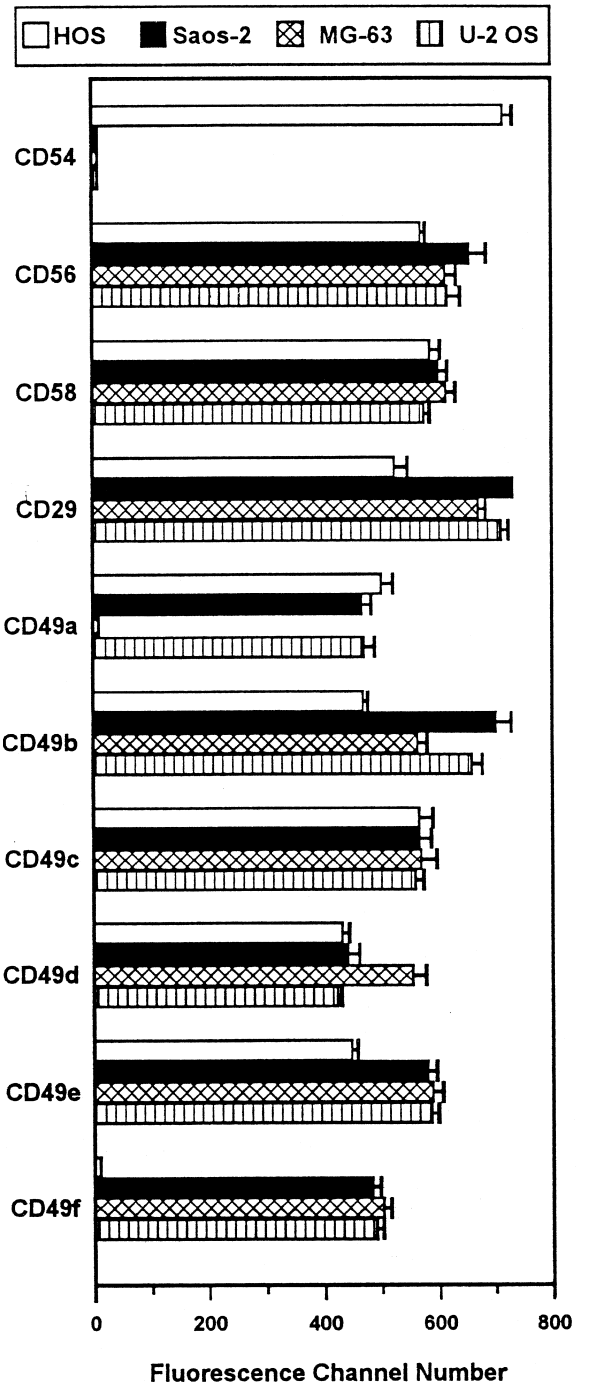

Fig. 2. Expression of adhesion molecules on osteosarcoma cell lines. Results are expressed as fluorescence channel number means ± S.E.M.

3. Results

3.1. Characterization of osteosarcoma cells

The susceptibility of the osteosarcoma cell lines to NK cell lysis varied considerably but NK cell lysis was greater in HOS cells than in the other lines (Fig. 1).

Cytofluorimetric analysis of the expression of adhesion molecules showed that only HOS cells were strongly positive for CD54 (Fig. 2) while CD58 and CD56, CD29 and CD49b–e were expressed similarly on all the cell lines. In contrast,

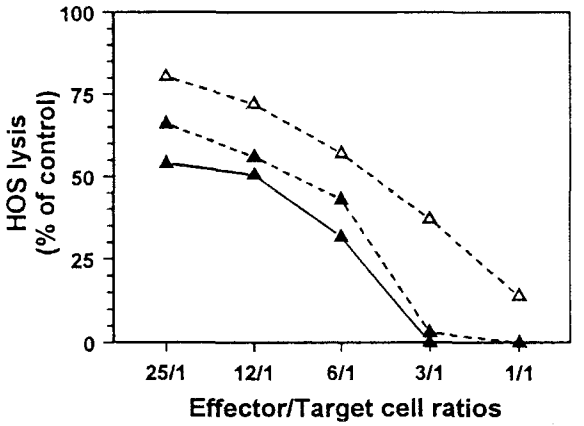

Fig. 3. Cytolytic activity of NK cells against HOS osteosarcoma cells after treatment with different concentrations of CD54 monoclonal antibody. Cytotoxicity assay was performed blocking CD54 on HOS cells with 4 μg/ml (closed triangles and continuous line); 0.2 μg/ml (closed triangles and dashed line); 0.08 μg/ml (open triangles and dashed line). Results are expressed as the percentage of control lysis mediated by unblocked cells (one representative experiment).

CD49a was present in all but MG-63 and CD49f in all but HOS cell lines (Fig. 2).

All cell lines were negative for CD102, CD50, CD11a, b, c/CD18 and CD2 (data not shown).

To establish the relevance of CD54 adhesion molecule expression to cytolytic reaction, HOS cells were incubated with different concentrations of CD54 blocking antibodies prior to co-culture with effector lymphocytes. The inhibition of NK cytolytic activity against HOS cells decreased progressively with the dilution of CD54 monoclonal antibody used for blocking target cells (Fig. 3). The saturation of the receptor using 4 μg/ml of CD54 was confirmed by flow cytometry: no changes in MESF values were observed with increasing doses (8 μg/ml) of blocking antibody. Therefore we used 4 μg/ml of CD54 for the following blocking tests.

In parallel experiments the physiological counter-receptor molecule (CD11a/CD18) was blocked on effector lymphocytes before incubation with untreated HOS cells that are CD54 negative. Cytolytic reaction was also performed co-culturing HOS target cells and lymphocytes after the treatment of each antigen on the respective cell.

A progressive inhibition of NK cell lysis was obtained in all three co-culture conditions (Fig. 4) and was more evident when both antigens were blocked on the respective cell and was complete at a 6/1 effector/target cell ratio.

3.2. Effect of TNFα incubation on osteosarcoma cells

TNFα incubation enhanced the susceptibility of the osteosarcoma cell lines to NK cell lysis (Fig. 5a–d) to different degrees. The increase was most evident on Saos-2 targets. The optimal dose of interleukin and the time required to induce a modified susceptibility to NK cell lysis were previously determined (data not shown).

To determine the effects of TNFα incubation on the expression of CD54 antigen, which seemed potentially involved in the regulation of susceptibility to NK lysis, the expression of this surface molecule was studied.

HOS ($CD54^+$) and Saos-2, MG-63, U-2 OS ($CD54^-$) osteosarcoma cells were treated with TNFα for 24 h. A consistent up-regulation of CD54 expression on HOS cells (Fig. 6a) (about twofold from 460 229 ± 57 924 to 1 059 380 ± 161 438 MESF, $P < 0.02$) and the expression of CD54 on the other osteosarcoma cell lines that did not constitutively present this molecule (MG-63: 332 033 ± 64 775 MESF; U-2 OS: 258 424 ± 73 449 MESF; Saos-2: 69 548 ± 11 563 MESF) was observed after TNFα treatment.

The consistent increment of CD54 intensity was correlated with a significant increase in the susceptibility to lysis of HOS cells after TNFα treatment ($P = 0.0001$, Fig. 7a). Also MG-63 U-2 OS (data not shown) and Saos-2 cells ($P = 0.005$, Fig. 7b) increased their susceptibility to lysis after TNFα treatment which was linked to the appearance of CD54 antigen (Fig. 6b), even if in Saos-2 cells the intensity of antigen expression was the lowest among the three CD54 osteosarcoma cells and more than 15-fold lower than in HOS cells.

To confirm whether the increased susceptibility to lysis by NK cells was a consequence of the increased expression of CD54 on TNFα-treated osteosarcoma cells, we selectively blocked this molecule on HOS and Saos-2 cells (selected because they represent the highest and lowest CD54 expression respectively). Treatment with CD54 antibody resulted in a significant inhibition of NK-mediated lysis of TNFα treated HOS and Saos-2 cells.

Almost equally effective was the treatment of the CD11a/CD18 counter-receptor molecule on effector lymphocytes, while combined treatment with CD54 and CD11a/CD18 on the corresponding cell further decreased susceptibility to NK cell lysis, which was inhibited by at least 80% at 3/1 effector/target cell ratio (data not shown).

These results confirm that the enhanced NK susceptibility of TNFα-treated HOS and Saos-2 cells depends on the increased or induced expression of CD54 antigen on target cells.

4. Discussion

This study shows that the different susceptibility of osteosarcoma cells to NK cell lysis is correlated with the different expression of the CD54 adhesion molecule.

We evaluated a large number of cell adhesion molecules including those previously described on osteosarcoma cells

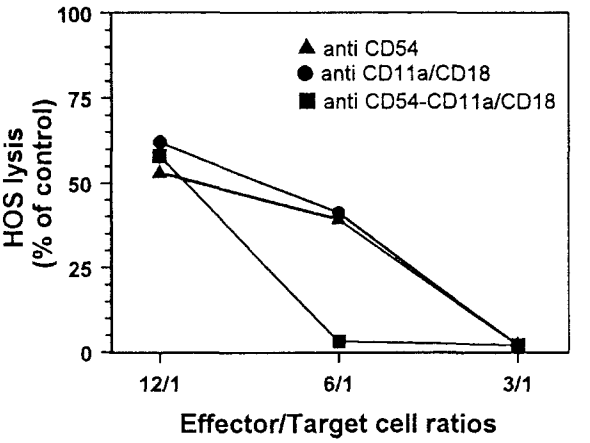

Fig. 4. Cytolytic activity of NK cells against HOS osteosarcoma cells after treatment with CD54 and/or CD11a/CD18 monoclonal antibodies. Cytotoxicity assay was performed blocking CD54 on HOS cells (▲), the counter-receptor CD11a/CD18 on lymphocytes (•) or both (■). Results are expressed as the percentage of control lysis mediated by unblocked cells (one representative experiment).

and those known to be important in spontaneous killing [18–21]. These molecules are involved in immune recognition and may play a role in host recognition of transformed cells.

We found that CD56 and CD58 are commonly expressed by osteosarcoma cells, so that variation in their expression does not seem to be related to differences in NK cell lysis susceptibility.

The VLA antigens were of greater potential interest since this receptor system may be activated on cytolytic effector cells and on tumor cells by interaction with extracellular matrix proteins such as laminin, collagen IV and fibronectin [22]. A variable expression of these antigens has been reported among tumor cell lines related to adhesive interaction with extracellular matrix and to tumor progression and spread [23]. In particular the absence of CD49a on MG-63 and CD49f on HOS may suggest a different ability of these two osteosarcoma cells to interact with collagen and laminin respectively.

No correlation of CD49d cell-mediated lysis susceptibility has been reported for different tumor cells [24,25]. The CD49d receptor does not seem fundamental in the interaction of bone-derived tumor cells with effector cells of the immune system for the development of a lytic response, because it is uniformly expressed on the osteosarcomas studied. A similarly uniform expression was also shown by HLA class I molecules (data not shown). Of all the receptors, only CD54 seems to be important in determining susceptibility to spontaneous NK cell lysis: the expression of CD54 correlated with NK susceptibility and the three tumor lines that were resistant to lysis were also all CD54-negative.

From our data it seems that low levels of CD54 expression are sufficient to achieve the susceptibility conferred by this ligand, since this molecule appeared to influence the vulnerability of the target cell line also at low levels of expression as in Saos-2 cells incubated with TNFα.

The importance of this molecule in determining susceptibility to NK cell lysis was confirmed by blocking studies, where the elimination of the CD54 cell surface molecule on target cells or its counter-receptor molecule (CD11a/CD18) on effector lymphocytes substantially inhibited NK lysis. These data are supported by several groups, which, in other experimental models, have described a marked inhibition of NK cell lysis by anti-CD54 and by anti-CD11a/CD18 monoclonal antibodies [24,25].

The poor inhibition with one or both antibodies at a 12/1 effector/target cell ratio can be related to the high number of effector cells, involved in the recognition of targets. At these high density cell conditions other surface receptors with low affinity and/or density can be involved in the binding of NK cells to osteosarcoma targets. At an effector/target ratio below

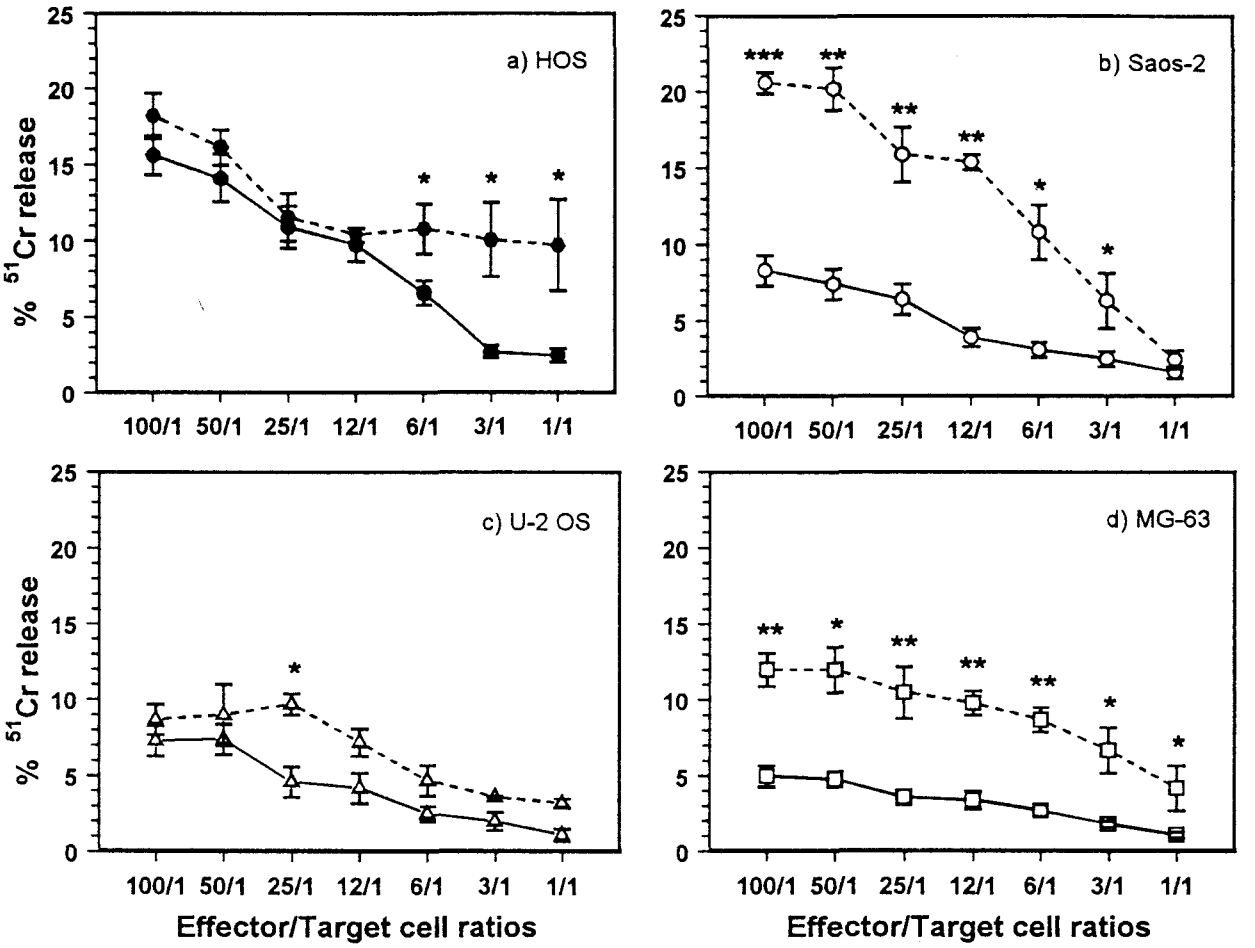

Fig. 5. Natural killer cell cytolytic activity against human osteosarcoma cell lines before (continous lines) and after TNFα incubation (dashed lines). Results are expressed as mean percentages ± S.E.M. Significant differences after TNFα treatment are indicated: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

12/1, which is more similar to the numerical ratios found in vivo, CD54 molecule expression seems to assume a predominant role in target cell recognition.

Furthermore, we demonstrated that incubation with TNFα up-regulated the CD54 antigen on HOS cells and induced the expression of this antigen on NK-resistant Saos-2 cells and in addition that CD54 modifications were correlated with the increase in target susceptibility to NK lysis. In these experimental conditions the block of CD54 or of its natural ligand on the respective cells confirmed the inhibition of lysis.

The relevance of this study to assess the susceptibility to NK cell lysis of fresh bone tumor cells depends on whether ligand expression in vivo is similar to that of the osteosarcoma cell lines examined. Some studies indicate that despite numerous differences human osteosarcoma cell lines can provide an experimental model for investigating specific aspects of bone cell function. In fact, the integrin profiles of some osteosarcoma cell lines are representative of the distribution of these molecules on bone cells and in particular the α4 subunit of β1 integrin (CD49d) expressed by all primary osteosarcoma cells [18,21]. Using this molecule, cells can adhere to the endothelial cell lining expressing VCAM-1 [26–28], penetrate through the endothelium, then extravasate to the parenchymal organs and form metastases. The CD49d/VCAM-1 pathway is the best characterized adhesion molecule pair involved in metastatic spread of some malignancies.

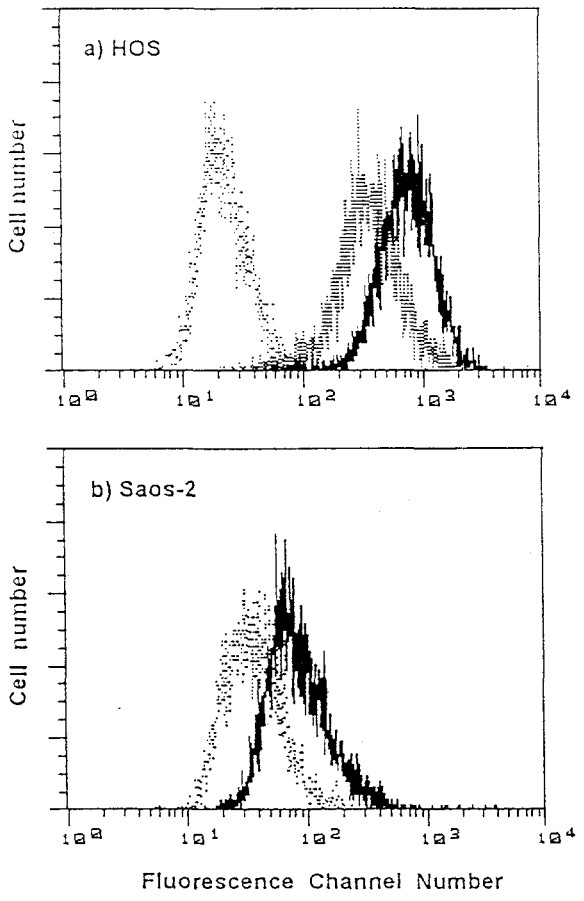

Fig. 6. Immunofluorescence analysis of CD54 on HOS (a) and Saos-2 (b) cell lines before (gray histograms) or after TNFα treatment (black histograms). Negative control is represented by the dotted histograms.

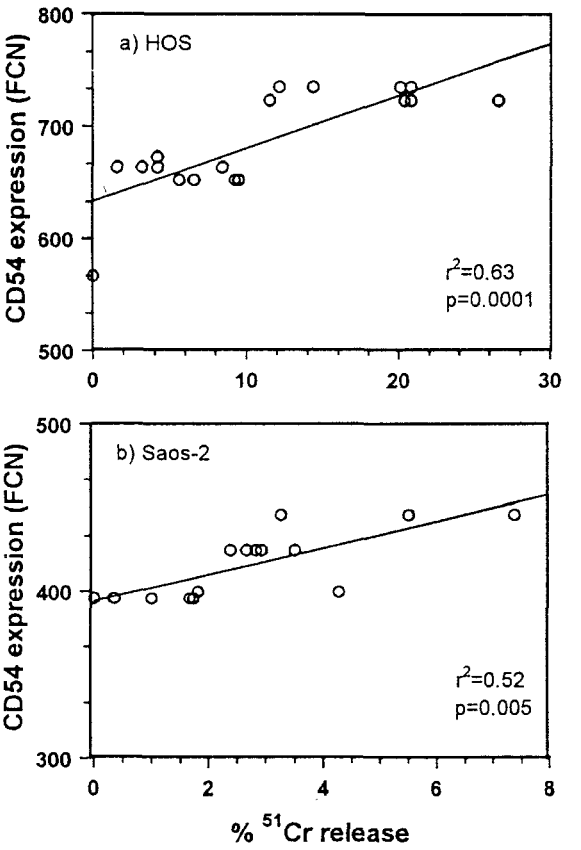

Fig. 7. Increased susceptibility to NK-mediated lysis of HOS (a) and Saos-2 (b) cell lines following TNFα treatment. Correlation between CD54 expression (FCN = fluorescence channel number) and percentage of ^{51}Cr release.

While there is no evidence that CD54 is expressed in clinical specimens of bone tumors, the expression of this molecule might correlate with a poor prognosis in melanomas [29].

Approximately 40% of patients with osteosarcoma tumor develop pulmonary metastases despite the administration of adjuvant chemotherapy, and the disease-free interval has not improved in recent years probably due to the presence of drug-resistant tumor cells. The development of new forms of tailor-made therapy is important in the hope of eradicating drug-resistant cells.

If the CD54 molecule also confers susceptibility to NK cell lysis in vivo and we can take advantage of local administration of TNFα for a positive modulation of the CD54 molecule, then the assessment of tumor susceptibility after exposure to chemotherapy would predict whether or not residual osteosarcoma cells would be susceptible to NK cell lysis and whether NK cells are candidates for adjuvant therapy.

Acknowledgements: This work was partially supported by grants from Progetti di Ricerca Corrente I.O.R. and M.U.R.S.T. 60%, Italy. The authors wish to thank Mrs. Anne Collins for revising the English of the manuscript, Mrs. Graziella Salmi and Patrizia Rappini for editorial assistance.

References

[1] Lewis, C.E. and McGee, J.O. (1992) The Natural Killer Cell. IRL Press, Oxford.
[2] H.G. Ljunggren, K. Karre, J. Exp. Med. 162 (1985) 1745–1759.

[3] G.E. Piontek, K. Taniguchi, H.G. Ljunggren, A. Groneberg, R. Kiessling, G. Klein, K. Karre, J. Immunol. 135 (1985) 4281–4288.
[4] A. Harel-Bellan, A. Quillet, C. Marchiol, R. Demars, T. Turst, D. Fradelizi, Proc. Natl. Acad. Sci. USA 83 (1986) 5688–5692.
[5] W.J. Storkus, D.N. Howell, R.D. Salter, J.R. Dawson, P. Cresswell, J. Immunol. 138 (1987) 1657–1659.
[6] W.J. Storkus, J. Alexander, J.A. Payne, P. Cresswell, J.R. Dawson, J. Immunol. 143 (1989) 3853–3857.
[7] M. Maio, M. Altomonte, R. Tatake, R.A. Zeff, S. Ferrone, J. Clin. Invest. 88 (1991) 282–289.
[8] G. Berke, Annu. Rev. Immunol. 12 (1994) 735–773.
[9] J.S. Rhim, H.Y. Cho, R.J. Huebner, Int. J. Cancer 15 (1975) 23–29.
[10] F. Jorgen, J.M. Fogh, T. Orfeo, J. Natl. Cancer Inst. 59 (1977) 221–225.
[11] A. Billiau, V.G. Edy, H. Heremans, J. Van Damme, J. Desmyter, J.A. Georgiades, P. De Somer, Antimicrob. Agents Chemother. 12 (1977) 11–15.
[12] J. Pontén, E. Saksela, Int. J. Cancer 2 (1967) 434–447.
[13] G. Weryha, J. Leclère, Horm. Res. 43 (1995) 69–75.
[14] M. Vitale, S. Papa, A.R. Mariani, A. Facchini, R. Rizzoli, F.A. Manzoli, J. Immunol. Methods 96 (1987) 63–68.
[15] E. Mariani, M.C.G. Monaco, L. Cattini, M. Sinoppi, A. Facchini, Mech. Ageing Dev. 76 (1994) 177–187.
[16] R.F. Vogt, G.D. Cross, L.O. Henderson, D.L. Phillips, Cytometry 10 (1989) 294–302.
[17] E. Mariani, M.C.G. Monaco, S. Sgobbi, J.F. De Swart, A.R. Mariani, A. Facchini, J. Immunol. Methods 172 (1994) 173–178.
[18] P. Mattila, M.L. Majuri, R. Renkonen, Int. J. Cancer 52 (1992) 918–923.
[19] S. Kawaguchi, T. Uede, J. Orthopaed. Res. 11 (1993) 386–395.
[20] R. Galandrini, N. Albi, R. Tognellini, A. Terenzi, E. Galluzzo, A. Tosti, D. Zarcone, C. Tenca, A. Velardi, FCI 1 (1993) 127–133.
[21] J. Clover, M. Gowen, Bone 15 (1994) 585–591.
[22] F. Mainiero, A. Gismondi, M. Milella, S. Morrone, G. Palmieri, M. Piccoli, L. Frati, A. Santoni, J. Immunol. 152 (1994) 446–453.
[23] S.M. Albelda, Lab. Invest. 68 (1993) 4–16.
[24] A. Anichini, R. Mortarini, R. Spino, G. Pariani, Int. J. Cancer 46 (1990) 508–513.
[25] N.K. Foreman, D.R. Rill, E. Coustan-Smith, E.C. Douglass, M.K. Brenner, Br. J. Cancer 67 (1993) 933–938.
[26] B.M.C. Chan, M.J. Elices, E. Murphy, M.E. Hemler, J. Biol. Chem. 267 (1992) 8366–8370.
[27] M.J. Elices, L. Osborn, Y. Takada, C. Crouse, S. Luhowskyj, M.E. Hemler, R.R. Lobb, Cell 60 (1990) 577–584.
[28] C. Ruegg, A.A. Postigo, E.E. Sikorski, E.C. Butcher, R. Pytela, D.J. Erle, J. Cell. Biol. 117 (1992) 179–189.
[29] P. Natali, M.R. Nicotra, R. Cavaliere, A. Bigotti, G. Romano, M. Temponi, S. Ferrone, Cancer. Res. 50 (1990) 1271–1278.