PERGAMON

European Journal of Cancer 39 (2003) 2395–2402

European Journal of Cancer

www.ejconline.com

Acquisition of anoikis resistance in human osteosarcoma cells

C.M. Díaz-Montero, B.W. McIntyre*

Department of Immunology, Unit 102, The University of Texas M.D. Anderson Cancer Center, PO Box 301402, Houston, TX 77030-1903, USA

Received 3 January 2003; received in revised form 14 March 2003; accepted 22 April 2003

Abstract

Under normal circumstances, adhered cells die of anoikis when detached from their extracellular matrix (ECM). Resistance to anoikis has been implicated in the progression of many human malignancies by affording an increased survival time in the absence of matrix attachment, facilitating the migration and eventual colonisation of distant sites. In this study, an anoikis-resistant variant of the human osteosarcoma cell line, SAOS-2 (SAOSar), was generated by sequential cycles of culturing under adhered and suspended conditions. It was also shown that although parental SAOS (SAOSp) cells are a heterogeneous population with varying levels of sensitivity to anoikis, the establishment of anoikis-resistant clones was not necessarily the result of mere selection of a previously resistant subpopulation. Anoikis-resistant cells were also derived from anoikis-sensitive SAOS clones by exposure to anoikis-inducing culture conditions. This suggests that lack of the normal signalling generated by attachment to the ECM could represent a driving force towards anoikis resistance. Resistance to anoikis could not be attributed to a general defect in the apoptotic pathway since apoptosis in both sensitive and resistant populations was induced after treatment with staurosporine, cycloheximide and hydrogen peroxide. This suggests that the apoptotic machinery is intact in both anoikis-sensitive and -resistant SAOS cells and that the death signal in anoikis-sensitive cells is generated by the lack of attachment, most probably by unligated integrins. Anoikis-resistant cells have circumvented this death signal and remain viable despite suspended conditions.

Keywords: Anoikis; Osteosarcoma; Integrins; ECM; SAOS; Cell adhesion

1. Introduction

Homeostasis in the normal tissue is achieved by a balance between cell proliferation and cell death that is reflected by an organised tissue architecture. When detached from the extracellular matrix (ECM), normal adherent cells undergo a form of apoptotic death known as anoikis. Anoikis, Greek for 'homeless', was first observed in primary cells from epithelial and endothelial origins in which apoptosis was rapidly induced when proper substrate adhesion was denied[1,2].

Attachment to the ECM is mainly mediated by integrins, a family of transmembrane proteins composed of an α and a β subunit [3–5]. Integrins do not possess kinase domains, but signal by associating with other molecules. In response to extracellular and intracellular physiological changes, integrins send bi-directional signals that in turn modulate cell proliferation, survival and migration [6–8].

Resistance to anoikis is known as one of the hallmarks of malignant transformation. It plays an important role during tumour progression by affording increased survival times in the absence of matrix attachment, facilitating migration and re-attachment, and therefore colonisation of secondary sites [9–11]. Overexpression of oncogenes such as *ras, raf, rac* and *src*, as well as the downregulation of suppressor genes such as *SHIP-2*, *PTEN*, and *p53* results in resistance to anoikis [12]. However the specific pathways involved are not yet completely elucidated.

Although resistance to anoikis has been described in many types of human malignancies, little is known of its role during the progression of osteosarcoma. This is of particular importance since 30–40% of osteosarcoma patients will develop lung metastasis despite aggressive chemotherapy and surgical resection of the primary lesion [13–15].

We previously demonstrated that parental SAOS human osteosarcoma cells are sensitive to anoikis [16].

* Corresponding author. Tel.: +713-563-3224; fax: +713-563-3351.

E-mail address: bmcintyr@mdanderson.org (B.W. McIntyre).

0959-8049/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0959-8049(03)00575-6

In the present study, we generated an anoikis-resistant variant from SAOSp cells by sequential cycles of culture under adherent and suspended conditions. It was unclear whether the resistant phenotype was due to clonal selection of a pre-existing subpopulation or the induction of a new differentiated state and we tested this in a series of cloning and induction experiments.

2. Materials and methods

2.1. Cell culture and reagents

The parental human osteosarcoma cell line, SAOS, was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). SAOSp cells were maintained in Eagle's minimum essential medium (EMEM) (BioWhittaker, Walkersville, MD, USA), supplemented with 10% fetal bovine serum (v/v) (BioWhittaker, Walkersville, MD, USA), 2 mM L-glutamine, 1 mM sodium pyruvate and non-essential amino acids (Sigma, St. Louis, MO, USA). Poly(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma, St. Louis, MO, USA) was prepared by dissolving it in 95% ethanol (v/v) to a concentration of 50 mg/ml. Poly-HEMA was added to cell culture wells at a density of 5 mg/cm^2. After poly-HEMA was added, wells were allowed to dry overnight, under sterile conditions in a laminar flow hood. Hydrogen peroxide (3% (w/w)) was purchased from Kroger. Staurosporine and cycloheximide were purchased from Sigma, St. Louis, MO, USA. Stock solutions of 1 mM and 1 mg/ml, respectively, were prepared in dimethylsulphoxide DMSO and kept frozen at −20 °C.

2.2. Generation of anoikis-resistant variants

Anoikis-resistant SAOS cells (SAOSar) were generated by sequential cycles of culturing on untreated (adhered) and poly-HEMA-treated cell culture wells (suspended). Briefly, SAOSp monolayers were trypsinised and transferred to poly-HEMA-treated wells. Cells were maintained under suspended conditions for the indicated time intervals. After incubation, cells were washed and transferred to untreated cell culture wells and allowed to replicate and to form monolayers. The resulting variants were maintained in culture under adhered conditions.

2.3. Generation of clonal populations from SAOSp cells

Single-cell clonal populations from SAOSp were generated by automated cell sorting (Beckman Coulter Epics ELITE, Beckman Coulter, Miami, FL, USA). Briefly, confluent monolayers of SAOSp cells were trypsinised, and single cells were deposited into separate wells of a 96-well cell culture plate. 100 μl of complete medium were added to each well. Plates were incubated at 37 °C in a 5% CO_2 atmosphere. Wells were monitored for the growth of single colonies. Single colonies were allowed to form confluent monolayers. Clonal populations were maintained under adherent conditions.

2.4. Cell-cycle analysis

Cell-cycle analyses were performed using propidium iodide (PI) staining with subsequent fluorescent activated cell sorting (FACS) analysis. 5×10^5 cells/well were cultured either on plastic or poly-HEMA-treated six-well tissue culture plates for 24 h at 37 °C in a 5% CO_2 atmosphere. After incubation, adherent cells were detached with trypsin (0.5% trypsin (w/v)/0.1% ethylene diamine tetraacetic acid (EDTA) (w/v) in phosphate-buffered solution (PBS)). Detached and suspended cells were harvested in complete EMEM medium and centrifuged at 500*g* for 10 min. Pellets were washed with PBS and fixed with ice-cold 75% ethanol (v/v) overnight at 4 °C. After fixation, cells were washed with PBS and stained with 500 μl of PI solution (50 μg/ml in PBS) containing 25 μg/ml of RNase. Cells were incubated at 37 °C for 30 min and analysed by flow cytometry on an Epics Profile flow cytometer (Coulter, Miami, FL, USA).

2.5. Cell viability assay

Confluent monolayers of SAOSp and anoikis-resistant SAOSar cells were grown on 96-well cell-culture plates. Monolayers were treated with the indicated doses of H_2O_2, staurosporine or cycloheximide for 24 h. After treatment, monolayers were washed with sterile PBS, and 50 μl of 2 μM Calcein-AM (Molecular Probes, Eugene, OR, USA) was added to each well. Plates were incubated at room temperature for 15 min, and fluorescence was determined using a Perkin-Elmer LS-50B luminescence spectrometer (Perkin-Elmer, Shelton, CT USA). The percentage of viable cells was calculated as follows:

$$\frac{\text{Experimental flourescence} - \text{Spontaneous flourescence}}{\text{Maximum flourescence} - \text{Spontaneous flourescence}} \times 100$$

3. Results

3.1. Culture of SAOSp in suspension triggers anoikis in a time-dependent manner

We previously demonstrated that SAOSp cells undergo anoikis after attachment to the ECM is prevented by culturing in poly-HEMA-treated culture wells

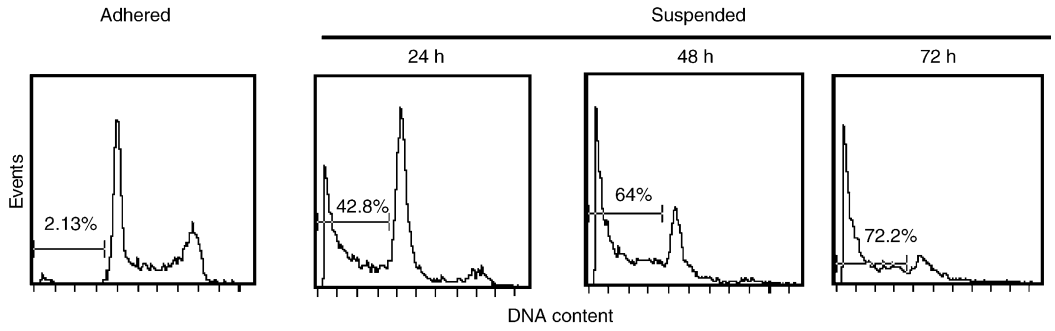

Fig. 1. Suspended parental SAOS (SAOSp) cells undergo anoikis in a time-dependent manner. SAOSp cells cultured under adherent conditions were trypsinised and added to untreated (adherent) or poly(2-hydroxyethyl methacrylate) (poly-HEMA)-treated (suspended) cell culture wells. After the indicated time intervals, cells were harvested and stained with propidium iodide (PI) as described in Materials and methods. PI staining was analysed by flow cytometry. The percentage of cells in the sub-G/0 phase representative of apoptotic cells is marked on each histogram. Data shown are representative of five independent experiments.

for 24 h [16]. In Fig. 1, the results of a time course experiment in which anoikis was assayed after several time intervals are shown. At 24 h, adherent SAOSp cells were alive (only 2.13% cell death was observed), but within the same time-frame under suspended conditions, 42.8% of the SAOSp cells died (Fig. 1). It was observed that anoikis of suspended SAOSp increased in a time-dependent manner reaching 72.2% cell death at 72 h of culturing (Fig. 1), and almost complete cell death after 6 days of culturing under suspended conditions (data not shown).

3.2. Culture of SAOSp cells under suspended conditions for 72 h increases their ability to resist anoikis

Since long periods of culture in suspension killed the parental SAOSp cells, we asked the question of whether resistance to anoikis of SAOSp cells could be achieved by a short sequential cycle of suspension culture followed by culture conditions that allowed normal attachment. SAOSp cells were cultured under suspended conditions (poly-HEMA-treated culture wells) for 72 h. After the 72-h incubation, cells were allowed to divide under adherent conditions until the monolayers were confluent, and the resulting sub-line was named SAOSar. The percentage of apoptosis for both SAOSp and SAOSar was determined after 24 h of culturing under either adhered or suspended conditions (Fig. 2). In both cell populations, a very low percentage (5.06% of SAOSp versus 5.89% of SAOSar) of apoptotic cells were found after culturing under adhered conditions. In contrast, fewer SAOSar than SAOSp cells underwent apoptosis after adherence was prevented (11.3% versus 34.8%, respectively). These results show that culturing of SAOSp under suspended conditions for 72 h results in a subline with an increased resistance to anoikis.

In contrast to the SAOSp population, in which no viable cells could be recovered after 6 days of culturing under suspended conditions, viable SAOSar cells were recovered after 14, 20, and even 30 days of culturing under suspended conditions. Moreover, when the resulting populations were recultured under normal adherent conditions and then reassayed for resistance to anoikis, all of them retained the ability to resist anoikis after culturing under suspended conditions for 24 h (Fig. 3). These results suggest that once a population becomes resistant to anoikis, the phenotype remains stable. More evidence for this stability comes from the fact that anoikis resistance is maintained in the SAOSar population even after 35 passages of *in vitro* culture under adhered conditions (data not shown).

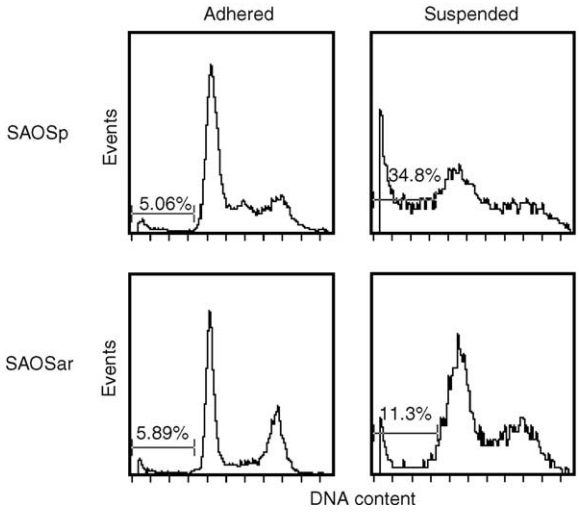

Fig. 2. Culture of parental SAOS (SAOSp) cells under suspended conditions for 72 h increases their ability to resist anoikis. SAOSp cells were maintained under suspended conditions for 72 hrs and then transferred to untreated tissue culture wells. After the monolayers became confluent, cells were harvested and cultured for 24 h under adhered or suspended conditions. After culturing, resistance to anoikis from the original parental population (SAOSp) and the resulting anoikis-resistant variant (SAOSar) was assayed by propidium iodine (PI) staining. Data shown are representative of more than five independent experiments.

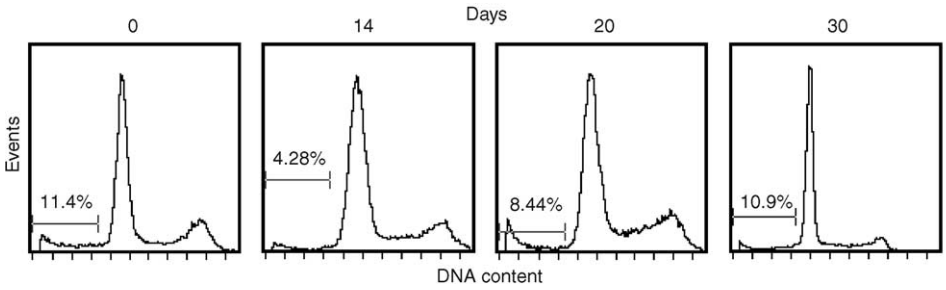

Fig. 3. Viable resistant SAOS-2 (SAOSar) cells could be recovered after long-term culture under suspended conditions. SAOSar cells were incubated on poly(2-hydroxyethyl methacrylate) (poly-HEMA)-treated plates for the indicated days. After culture, cells were transferred to untreated tissue culturing wells and allowed to form a confluent monolayer. Anoikis resistance of the resulting sublines was assayed after 24 h of culturing under suspended conditions by propidium iodine (PI) staining. Data shown are representative of at least three independent experiments.

3.3. SAOSp cells are a heterogeneous population with different levels of anoikis resistance

Tumours are characterised by being heterogeneous, potentially containing cells with vastly different phenotypes. Our treatment of SAOSp cells with anoikis-inducing conditions may have resulted in the survival of resistant cells already present within the heterogeneous parental population. Therefore, we wanted to determine whether anoikis resistance was the result of clonal selection. For this purpose, we generated single-cell clonal populations from SAOSp using automated cell sorting. Clonal populations were allowed to expand under adhered conditions, and their ability to resist anoikis was assayed after 24 h of culturing under adhered and suspended conditions (Fig. 4). Low background apoptotic death was observed after culturing under adhered conditions, confirming a similar cell viability among all the clones obtained. In contrast, different levels of apoptosis were found after culturing under suspended conditions for 24 h ranging from 60.4 to 8.2% (mean = 34.99%, Standard Deviation (S.D.) = 16.90%). Although most of the clones (86.4%) were found to be sensitive to anoikis, clones $SAOS_{c13}$ and $SAOS_{c5}$ were relatively resistant to anoikis. Relative resistance to anoikis was defined as the percentage of apoptosis below 1 S.D. of the mean.

The fact that two clones were found to be relatively resistant to anoikis with apoptotic levels similar to the ones found in SAOSar cells suggests the possibility that this anoikis-resistant population could have been obtained by selection of anoikis-resistant cells constitutively present among the parental population.

To test this hypothesis, seven SAOSp clones with different levels of sensitivity to anoikis were cultured under suspended conditions for 72 h. Viable cells were allowed to divide under adhered conditions. Recovered cells were then incubated for 14 days under suspended conditions. After incubation, cells were allowed to expand under adhered conditions. After viable cells formed a confluent monolayer, cells were tested for their ability to resist anoikis after 24 h of culture under suspended conditions. Results in Fig. 5 represent the percent change in apoptosis from the original clone versus its corresponding variant obtained after the two cycles of anoikis-inducing culture. Data are shown for only six out the seven clones chosen because no viable cells from $SAOS_{c2}$ were recovered after the 14-day culture under suspended conditions. No significant change in resistance was observed among the $SAOS_{c4}$ cells. Anoikis resistance was maintained in the clone $SAOS_{c13}$ that was originally resistant to anoikis. Interestingly, clones $SAOS_{c1}$, $SAOS_{c3}$ and $SAOS_{c9}$ had an increased resistance to anoikis with a percentage reduction in apoptotic death of 58, 59 and 59%, respectively. These results suggest that clonal selection is not the only driving force in anoikis resistance, and that differentiation towards a resistant phenotype could be driven by exposure to anoikis-inducing culture conditions. Thus, these results are not due to the mere selection of pre-existing resistant sub-populations.

3.4. Resistance to anoikis in SAOS cells is not due to a generalised defect in the apoptotic pathway

A crucial acquired capability of tumour cells is evasion of apoptosis. This can be accomplished through a variety of ways, the most commonly being loss of tumour suppressor genes. Since SAOSp cells lack both the *p53* and *Rb* genes [17,18], we wanted to determine if resistance to anoikis was the result of a generalised defect in the apoptotic machinery that rendered the cells resistant to any apoptotic stimuli. Since viable SAOSar cells recovered after incubation cycles of 72 h and 14 days showed the highest anoikis resistance (Fig. 3) this subline, SAOSar14d, was used. Results shown in Fig. 6 demonstrate that apoptosis in SAOSp cells is triggered by oxidative damage (H_2O_2) and the inhibition of both protein synthesis (cycloheximide) and calcium-dependent protein kinases (staurosporine). The fact that SAOSar14d cells behave the same way indicated that the apoptotic machinery is intact and functional in anoikis-resistant cells as well. These findings were corroborated using PI staining followed by flow cytometry

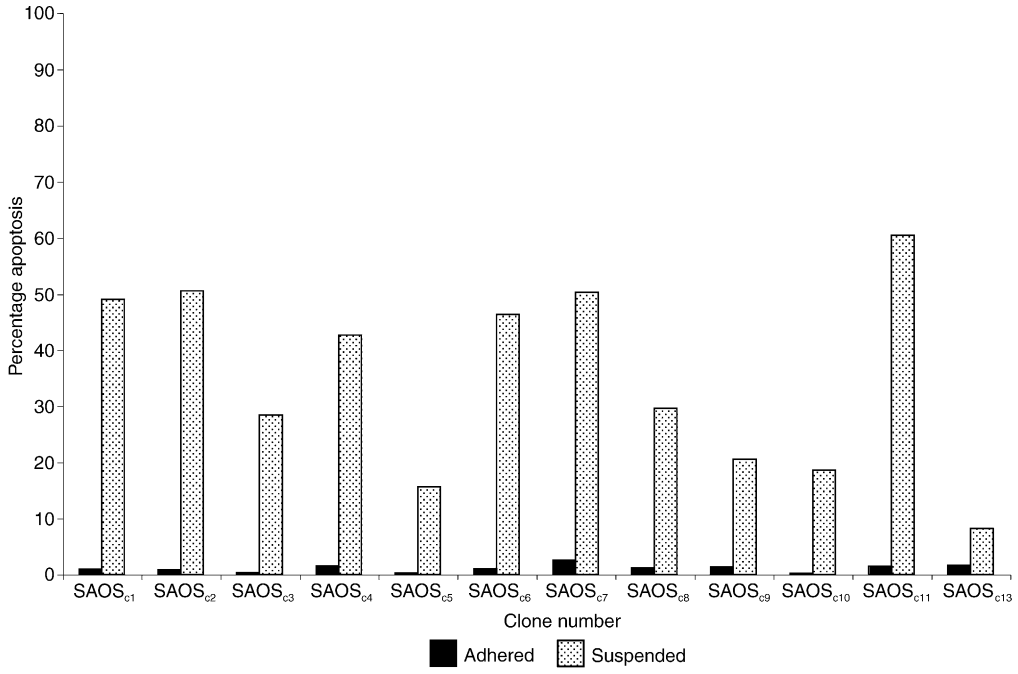

Fig. 4. Parental SAOS (SAOSp) cells are a heterogeneous population with different levels of anoikis resistance. SAOSp cells were separated into single cells fractions by cell sorting, and then allowed to expand under adherent conditions. The ability to resist anoikis was assayed by propidium iodine (PI) staining in each clonal population after culturing for 24 h under adhered and suspended conditions. Data shown are representative of at least three independent experiments.

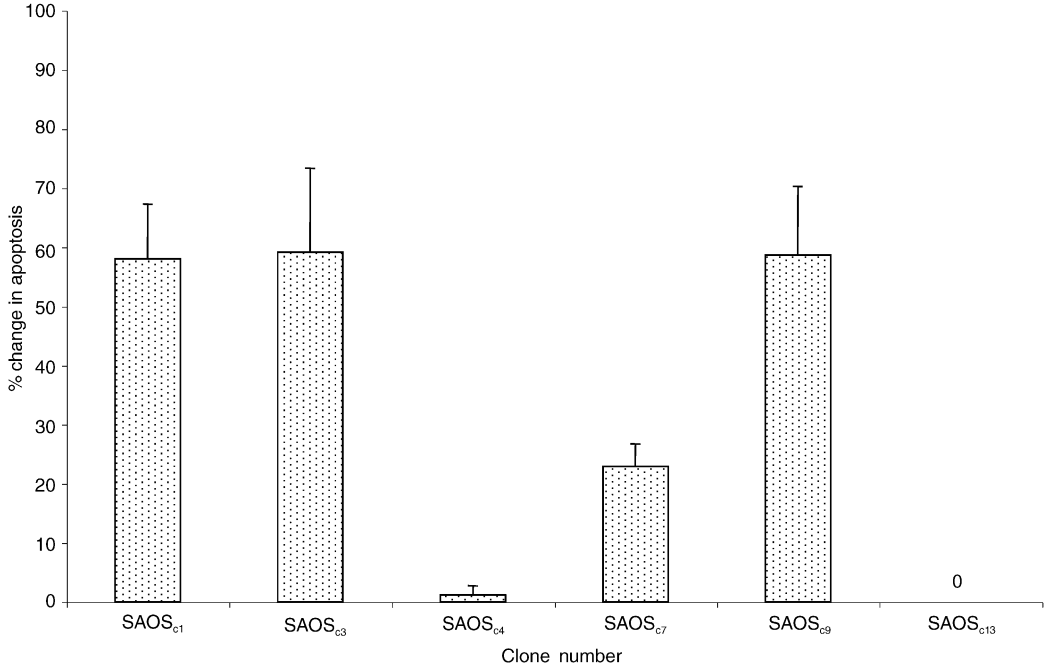

Fig. 5. Induction of anoikis-resistant cells. Parental SAOS (SAOSp) clonal populations were cultured under suspended conditions for 72 h, allowed to divide under adherent conditions, and cultured again under suspended conditions for 14 days. Viable cells were then allowed to multiply under adherent conditions. Resistance to anoikis was assayed after culturing under non-adherent conditions for 24 h. Results are expressed as the−% change in apoptosis, calculated as: (% apoptosis parental clone−% apoptosis resulting clone/% apoptosis parental clone) × 100. Error bars indicate the standard deviation from three independent experiments.

analyses (data not shown). Indeed, apoptosis was induced at similar levels in SAOSp, SAOar14d cells, three clones ($SAOS_{c4}$, $SAOS_{c7}$ and $SAOS_{c13}$) and their respective sublines generated after two cycles of culturing under suspended conditions. $SAOS_{c13}$ was originally resistant to anoikis and the resulting subline retained this ability. Clone $SAOS_{c7}$ was initially sensitive and the resulting subline became resistant to anoikis. Clone $SAOS_{c4}$ was initially sensitive and the resulting subline remained sensitive. These results suggest that apoptosis can be induced at similar levels in both sensitive and resistant populations, regardless of the fact that the resistant phenotype was already present in the population or was induced by culturing under suspended conditions.

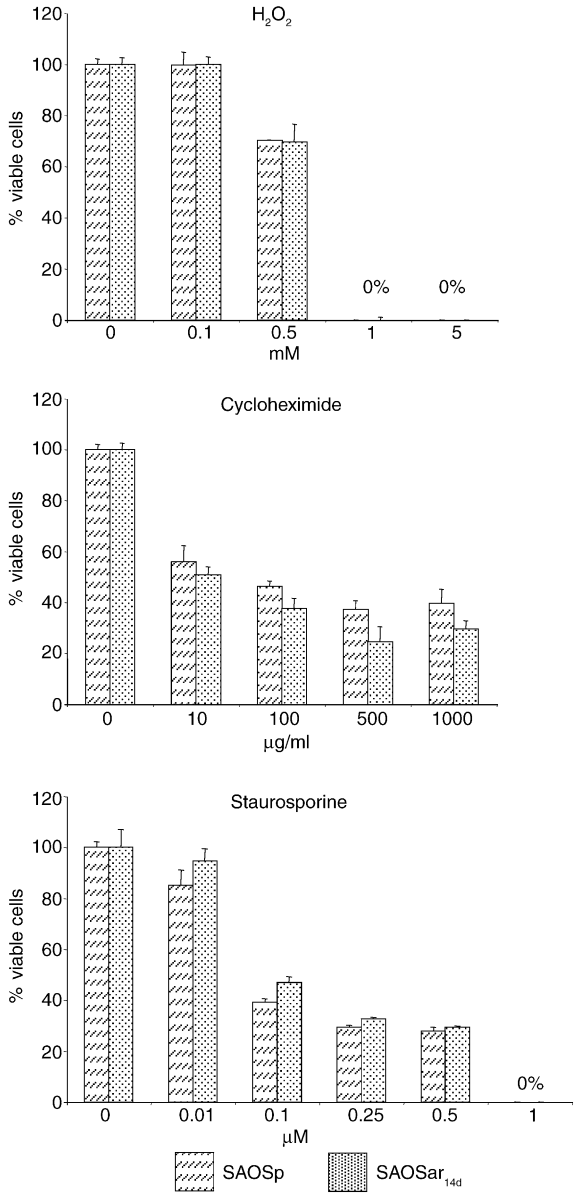

Fig. 6. The apoptotic machinery is intact in anoikis-resistant SAOS-2 (SAOSar) cells. Apoptosis in both anoikis-resistant ($SAOSar_{14d}$) and sensitive (parental SAOS (SAOSp)) cells was triggered by H_2O_2-induced oxidative damage (top), by cycloheximide-induced inhibition of protein synthesis (middle) and by staurosporine-induced inhibition of calcium-dependent protein kinases (bottom). Results are shown as percentage viable cells after the indicated treatments. Error bars represent the standard deviation of triplicate measurements. Data shown are representative of three independent experiments.

4. Discussion

Anoikis has evolved as a mechanism by which the organism ensures the proper development and maintenance of its tissue architecture. Not surprisingly, the breakdown of anoikis contributes to the progression of many human malignancies.

In this study, we have analysed the sensitivity of SAOSp osteosarcoma cells to anoikis. We showed that anoikis of SAOSp cells increases with time, reaching almost complete death after culturing under anoikis-inducing conditions (poly-HEMA-treated wells) for more than 6 days. Nevertheless, an anoikis-resistant subline, SAOSar, was developed after culturing for 72 h under suspended (anoikis-inducing) conditions. Interestingly, once this resistant line was established, we were able to recover viable SAOSar cells after 30 days of culturing in suspension. Furthermore, we observed that SAOSar cells remained anoikis-resistant after at least 35 passages of *in vitro* culturing (data not shown). This indicates that once the resistant phenotype was acquired, it remained stable.

Anoikis resistance of SAOSar cells could not be attributed to a generalised defect in the apoptotic pathway. Both anoikis-sensitive and -resistant SAOS cells were equally sensitive to apoptosis after treatment with staurosporine, cyclohexamide and H_2O_2. This suggests that what protects SAOSar cells from apoptosis is their ability to circumvent the death signals generated, most likely by integrins, in response to the lack of attachment.

Many phenotypic changes could contribute to the resistance to anoikis. Upregulation of oncogenes and downregulation of tumour suppressor genes are by far the most studied [19–23]. In addition, other phenotypic characteristics such as increased metastatic and angiogenic potentials have been described, not only as the result of anoikis resistance, but also as collateral capabilities that synergistically contribute to the progression of tumours [24–27].

It is still unknown whether anoikis resistance is the result of an accumulation of mutations that are selected when the cells detach from the ECM, or if it is an acquired phenotype driven by the lack or acquisition of signalling generated during attachment to the ECM. Our initial results suggested that anoikis resistance was the result of clonal selection of an already pre-existing resistant population. However, the fact that populations from a single-cell origin, originally sensitive to anoikis, became resistant after culturing under suspended

conditions suggests that different mechanisms other than mere clonal selection were involved in the generation of anoikis-resistant populations.

The recently revalidated field of epigenetics may provide a plausible explanation for this phenomenon [28–30]. Non-genetic changes in the patterns of gene expression due to gene silencing, reprogramming or imprinting in response to the lack of attachment could be responsible, at least in part, for the generation of anoikis-resistant cells. Breast cancer cells can overcome senescence and become immortalised by silencing the cyclin-dependent kinase inhibitor $p16^{INK4a}$ through methylation of its promoter [31,32]. Gene silencing can also be achieved through posttranscriptional RNA interference. *Bcl-2* in human prostate cancer LNCaP cells is successfully silenced after transfection with complementary RNA/DNA oligonucleotide constructs, increasing their susceptibility to apoptosis [33].

Since malignant phenotypes are associated with multiple, rather than single, genetic and epigenetic changes, the acquisition of anoikis resistance might be the result of a combination of both. Oncogene activity can promote genetic instability [34], thereby creating a permissive scenario for epigenetic changes, which in our model could result in anoikis resistance. The model we have generated in these studies clearly points to the differentiation of osteosarcoma cells driven by de-adhesion. Our future studies will focus on identifying the molecular and genetic changes that mediate the differentiation of anoikis-sensitive osteosarcoma cells to the resistant phenotype.

Acknowledgements

This work was supported by the NIH grants CA 62596 and CA 166672 Cancer Center Support Core Grant. C. Marcela Díaz-Montero is the recipient of the Onstead Foundation Fellowship for Osteosarcoma Research.

References

1. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 1994, **124**, 619–626.
2. Meredith Jr. JE. The extracellular matrix as a cell survival factor. *Mol Biol Cell* 1993, **4**, 953–961.
3. Brakebusch C, Bouvard D, Stanchi F, Sakai T, Fassler R. Integrins in invasive growth. *J Clin Invest* 2002, **109**, 999–1006.
4. Ruoslahti E, Hayman EG, Pierschbacher MD. Extracellular matrices and cell adhesion. *Arteriosclerosis* 1985, **5**, 581–594.
5. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992, **69**, 11–25.
6. Miyamoto S, Teramoto H, Coso OA, *et al*. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J Cell Biol* 1995, **131**, 791–805.
7. Giancotti FG, Ruoslahti E. Integrin signaling. *Science* 1999, **285**, 1028–1032.
8. Meredith Jr. JE, Winitz S, Lewis JM, *et al*. The regulation of growth and intracellular signaling by integrins. *Endocr Rev* 1996, **17**, 207–220.
9. Shanmugathasan M, Jothy S. Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. *Pathol Int* 2000, **50**, 273–279.
10. Streuli CH, Gilmore AP. Adhesion-mediated signaling in the regulation of mammary epithelial cell survival. *J Mammary Gland Biol Neoplasia* 1999, **4**, 183–191.
11. Yawata A, Adachi M, Okuda H, *et al*. Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells. *Oncogene* 1998, **16**, 2681–2686.
12. Grossmann J. Molecular mechanisms of "detachment-induced apoptosis-anoikis". *Apoptosis* 2002, **7**, 247–260.
13. Ferguson WS, Goorin AM. Current treatment of osteosarcoma. *Cancer Invest* 2001, **19**, 292–315.
14. Goorin AM, Shuster JJ, Baker A, Horowitz ME, Meyer WH, Link MP. Changing pattern of pulmonary metastases with adjuvant chemotherapy in patients with osteosarcoma: results from the multiinstitutional osteosarcoma study. *J Clin Oncol* 1991, **9**, 600–605.
15. Link MP, Goorin AM, Miser AW, *et al*. The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. *N Engl J Med* 1986, **314**, 1600–1606.
16. Marco RAW, Díaz-Montero CM, Wygant JN, Kleinerman ES, McIntyre BW. α4 Integrin increases anoikis of human osteosarcoma cells. *J Cell Biochem* 2003, **88**, 1038–1047.
17. Chandar N, Billig B, McMaster J, Novak J. Inactivation of p53 gene in human and murine osteosarcoma cells. *Br J Cancer* 1992, **65**, 208–214.
18. Dittmer D, Pati S, Zambetti G, *et al*. Gain of function mutations in p53. *Nat Genet* 1993, **4**, 42–46.
19. McFall A, Ulku A, Lambert QT, Kusa A, Rogers-Graham K, Der CJ. Oncogenic Ras blocks anoikis by activation of a novel effector pathway independent of phosphatidylinositol 3-kinase. *Mol Cell Biol* 2001, **21**, 5488–5499.
20. Rosen K, Rak J, Leung T, Dean NM, Kerbel RS, Filmus J. Activated Ras prevents downregulation of Bcl-X(L) triggered by detachment from the extracellular matrix. A mechanism of Ras-induced resistance to anoikis in intestinal epithelial cells. *J Cell Biol* 2000, **149**, 447–456.
21. Frisch SM, Vuori K, Kelaita D, Sicks S. A role for Jun-N-terminal kinase in anoikis; suppression by bcl-2 and crmA. *J Cell Biol* 1996, **135**, 1377–1382.
22. Zugasti O, Rul W, Roux P, *et al*. Raf-MEK-Erk cascade in anoikis is controlled by Rac1 and Cdc42 via Akt. *Mol Cell Biol* 2001, **21**, 6706–6717.
23. Vitale M, Di Matola T, Bifulco M, Casamassima A, Fenzi G, Rossi G. Apoptosis induced by denied adhesion to extracellular matrix (anoikis) in thyroid epithelial cells is p53 dependent but fails to correlate with modulation of p53 expression. *FEBS Lett* 1999, **462**, 57–60.
24. Zhu Z, Sanchez-Sweatman O, Huang X, *et al*. Anoikis and metastatic potential of cloudman S91 melanoma cells. *Cancer Res* 2001, **61**, 1707–1716.
25. Mahoney MG, Simpson A, Jost M, *et al*. Metastasis-associated protein (MTA)1 enhances migration, invasion, and anchorage-independent survival of immortalized human keratinocytes. *Oncogene* 2002, **21**, 2161–2170.
26. Fiucci G, Ravid D, Reich R, Liscovitch M. Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. *Oncogene* 2002, **21**, 2365–2375.
27. Rak J, Mitsuhashi Y, Sheehan C, *et al*. Collateral expression of proangiogenic and tumorigenic properties in intestinal epithelial

cell variants selected for resistance to anoikis. *Neoplasia* 1999, **1**, 23–30.
28. Belinsky SA. Epigenetics is alive and growing. *Trends Mol Med* 2002, **8**, 53–54.
29. Urnov FD, Wolffe AP. Above and within the genome: epigenetics past and present. *J Mammary Gland Biol Neoplasia* 2001, **6**, 153–167.
30. Barcellos-Hoff MH. It takes a tissue to make a tumor: epigenetics, cancer and the microenvironment. *J Mammary Gland Biol Neoplasia* 2001, **6**, 213–221.
31. Huschtscha LI, Noble JR, Neumann AA, *et al*. Loss of p16INK4 expression by methylation is associated with lifespan extension of human mammary epithelial cells. *Cancer Res* 1998, **58**, 3508–3512.
32. Foster SA, Wong DJ, Barrett MT, Galloway DA. Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Mol Cell Biol* 1998, **18**, 1793–1801.
33. Lin SL, Chuong CM, Ying SY. A Novel mRNA-cDNA interference phenomenon for silencing bcl-2 expression in human LNCaP cells. *Biochem Biophys Res Commun* 2001, **281**, 639–644.
34. Jain M, Arvanitis C, Chu K, *et al*. Sustained loss of a neoplastic phenotype by brief inactivation of MYC. *Science* 2002, **297**, 102–104.