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Hyperthermia triggers apoptosis and affects cell adhesiveness in human neuroblastoma cells

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Summary. Hyperthermia is a known apoptotic inducer and has been recently utilized in combination with chemo-and/or radiotherapy in cancer treatment. In this study we have described its effect on SK-N-MC human neuroblastoma tumor cells, a line which grows as a double adherent and floating population. Considering this particular culture behavior, we also investigated the relationship between hyperthermia and cell adhesiveness by evaluating integrin expression, namely CD11a, which is, as known, closely correlated to cell adhesion properties. By a multiple, ultrastructural and flow cytometrical approach, we have demonstrated that hyperthermia, while triggering apoptosis, also determines a CD11a surface expression decrease in apoptotic and living cells. We thus suggest a further role for this treatment, which, affecting adhesion mechanisms, could down-regulate metastatic diffusion.

Key words: SK-N-MC cell hyperthermia, Apoptosis, Integrins, Ultrastructure, Flow cytometry

Introduction

Apoptosis is a highly conserved process of physiological cell death, required for normal development and adult tissue homeostasis (Ioza et al., 2002). Abnormalities in cell death control are implicated in the pathogenesis of a number of diseases, including degenerative disorders and cancer (Jellinger, 2001; Green, 2002). In this respect, apoptosis is considered an essential cell response to agents used for anti-neoplastic chemotherapy and radiotherapy. Its role in the control of primary tumor mass has been widely described and a large number of investigations are in progress to clarify the apoptotic cellular response to newly-synthesized chemicals or to recently standardized physical treatments (Teh et al., 2002).

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Nevertheless, its correlation with the control of metastatic diffusion is only partially known (Azuma et al., 2002). It has been reported that tumor cell spreading is mostly due to the presence of trans-membrane proteins which mediate the relationship between cell and extracellular matrix (ECM) (Skubitz, 1992; Tzinia et al., 2002). Many of these interactions are mediated by integrins, a group of ubiquitously expressed adhesion receptors which bind to ECM -in particular collagens and laminins- on the outer cell side, and to particular cytoplasmic components -actin, vinculin, α–actinin-, inside the cell (Brakebusch et al., 2002).

Integrins are components of both "outside-in" and "inside-out" signalling systems. Through the first pathway, binding of ECM proteins to integrins alters gene expression and affects cellular proliferation and differentiation. Moreover, they are functionally correlated to messanger molecules active in signal transduction (Katagiri et al., 2000; Danilkovitch-Miagkova et al., 2002), thus regulating a variety of biochemical pathways.

The "inside-out" signal transduction is important for leukocyte recruitment during inflammatory responses, because conformational changes in the integrin heterodimer are induced by leukocyte signals and substantially increase leukocyte adhesiveness to the endothelial layer (Etzioni, 1999).

Integrins are thus involved in inflammation and immunity (Hanayama et al., 2002; Wang et al., 2002; Wright et al., 2002), as well as in cell migration, essential not only for tissue infiltration and metastatic diffusion, but also in physiological processes, such as angiogenesis (Kronenwett et al., 2002; Ribatti et al., 2002), leukocyte extravasation (Weber et al., 2001) and platelet aggregation (Mine et al., 2001). In addition, they take part in metabolic disorders such as osteoporosis or neurodegenerative diseases (Mousa , 2002).

The first event in cell migration is cell polarization, with the formation of a membrane protrusion at the leading edge. Integrins fix cell protrusions to ECM (Kabir et al., 2002), interact with actin and trigger the association of many different signaling molecules at the so-called focal contacts (Kornberg, 1998). The rear of

the cell detaches from the substratum as a consequence of integrin inactivation and adhesion complex disassembly (Weyant et al., 2000).

Integrin-mediated cell-matrix adhesion and the consequent cell movement, are enhanced by the recently described cooperation between adhesion molecules and matrix serine-proteases and metallo-proteases (Gazzanelli et al., 2000; Del Rosso et al., 2002; Tzinia et al., 2002). Differently, when integrin-mediated cell-ECM interaction is lost, apoptosis, or, precisely, an unusual phenomenon named "anoikis" can occur (Frisch and Ruoslahti, 1997; Bonfoco et al., 2000; Frisch and Screaton, 2001).

Integrin molecule is formed by two subunits, α and ß coupled by non-covalent bindings (Fig. 1). 15 α and 8 ß subunits have been actually identified, which can combine to yield at least 24 different integrin heterodimers, each with distinct ligand binding and signaling properties.

In the ß2 integrin subfamily, ß subunit can combine with four different α chains, known as CD11a, CD11b, CD11c and CD11d. The complex CD11a/CD18 (also named LFA-1, Leukocyte Function-Associated molecule-1) is a 275 kDa integrin receptor, mostly expressed in white blood cells, where it plays a role in cell-cell and leukocyte-endothelium interactions. Moreover, its importance has been described in development (Tani-Ishii et al., 2002) and in microglia (Mizuno et al., 1999), where it mediates cell-cell interactions with telencephalic neurons (Bonfoco et al., 2000).

The purpose of the present work is to investigate the behavior of LFA-1 in experimental tumor cell adhesion and its correlation with apoptosis. We chose SK-N-MC cells, a human neuroblastoma line, normally growing as a double, adherent and floating, population. In our opinion, this model represents a convenient system for studying the correlations among apoptosis, cell shape, ahesion effectiveness and integrin expression, and they have been investigated by a multiple, cytometrical and ultrastructural approach.

Materials and methods

Cell culture and treatment

SK-N-MC human neuroblastoma cell line was cultured in RPMI 1640 (Seromed, Biochrom., Berlin, Germany) supplemented with 2 mM glutamine, 25 mM Hepes pH 7.5, 1% antibiotics and 1% non-essential aminoacids. It was then maintained in a 5% CO_2 atmosphere at 37 °C and cell viability was assessed by the Trypan Blue exclusion test (Luchetti et al., 2002).

For the induction of apoptosis, hyperthermia was utilized, in terms of 43 °C treatment in a water bath for 1 h, followed by a 37 °C post-incubation for 5 h in 5% CO_2 atmosphere (Falcieri et al., 2000a,b).

Adhesion effectiveness of floating cells was also investigated.

For this purpose, suspension cells from control and hyperthermic specimens were withdrawn and seeded in new flasks. Incubation was carried out for 24 h in standard conditions, as described above.

Flow cytometry

Adherent and floating SK-N-MC cells were analysed separately. Controls and specimens undergoing hyperthermia from both populations, were examined for reactivity against CD11a using a direct immunostaining, carried out with an anti CD11a FITC-conjugated monoclonal antibody (Ancell, clone 38, Bayport, MN, USA), vs a FITC-conjugated isotypic control.

Staining was performed using the appropriate volume of mAb and cells ($0.5x10^6$) were suspended in 50 μl of PBS, supplemented with 5% of human plasma, to reduce aspecific binding.

Samples (without PI) were incubated for 15 min at room temperature, in the dark. They were then washed in PBS and centrifuged for 8 min at x1200 g, supernatant was discarded and cellular pellets were disrupted and resuspended in 300 μl of PBS.

Specimens were then analysed, within 1 hour, with a FACScan cytofluorimeter (BD, Palo Alto, CA, USA) equipped with an argon ion laser tuned at 488nm.

To simultaneously evaluate apoptotic cells, necrotic ones and CD11a expression, we performed an identical staining on SK-N-MC cells, freshly incubated with propidium iodide (PI). The fluorochrome was added to the cells at a final concentration of 50 μg/ml, for 30 min, before final flask washing steps. This method allowed the identification of early apoptotic cells, which characteristically showed, an initial and progressive permeability alteration, even if in the presence of an apparent membrane structural integrity (Zamai et al., 2001).

Adherent and floating cells were then labelled as mentioned above. Samples (with PI) were then analysed within 1 hour with the FACScan cytofluorimeter, by acquiring not less than 5000 events for each tube.

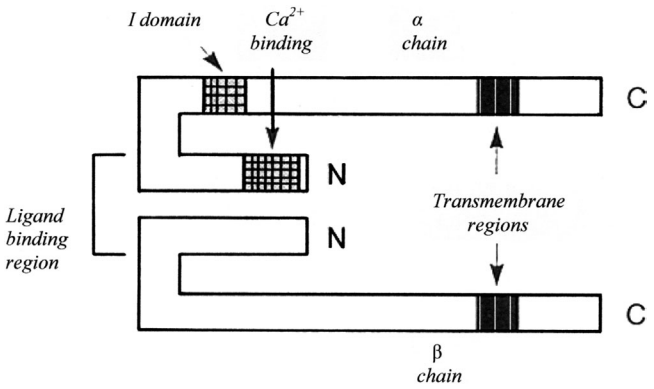


Fig. 1. Basic integrin structure.

To analyse DNA content of floating and adherent SK-N-MC cells, samples were also fixed by cold ethanol. They were then maintained for at least 30 min at 4 °C and washed two times in PBS.

Cell pellets were resuspended in citrate buffer, PI (25 μg/ml) and RNase (100 μg/ml), and finally maintained at 37°C for at least 30 min (Ormerod, 1994) before the cytometric acquisition.

Electron microscopy

For transmission electron microscopy (TEM) floating and adherent SK-N-MC cells were separately processed, both in normal condition and after hyperthermia. The first were collected, quickly washed and pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h.

OsO_4 post-fixation, alcohol dehydratation and araldite embedding were performed as previously described (Marini et al., 2001).

The adherent cells were washed, immediately fixed with 2.5% glutaraldehyde for 5 min, gently scraped and centrifuged at 1200 rpm. Pellets were additionally fixed for 1 h and processed as above.

This procedure allowed a good maintenance of the cell shape and prevented TEM of cell monolayers which, even if possible, provides limited information and is difficult to carry out.

Thin sectioning was preceded by semithin section analysis (Falcieri et al., 2000a), which allowed an overall specimen view.

Thin sections, collected on nickel grids, were stained with uranyl acetate and lead citrate and analysed with a Philips CM 10 electron microscope.

For scanning electron microscopy (SEM), control and hyperthermic cells were quickly washed and fixed "in situ" with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h.

After OsO_4 post-fixation, they were alcohol dehydrated and critical-point dried, as previously reported (Falcieri et al., 2000b). After mounting on microscope stubs, followed by gold sputtering, specimens were observed with a Philips 515 scanning electron microscope.

Results

Ultrastructural morphology was firstly characterized by TEM (Fig. 2) in both cellular populations.

Adherent cells appeared elongated and showed large, ovoidal nuclei, with prominent nucleoli (Fig. 2A). Floating cells, withdrawn from culture surnatant, evidenced a rounded shape and an apparently smaller nuclear-cytoplasmic ratio. Occasionally, within the floating cell population, mitoses could revealed (Fig. 2B).The insets display the corresponding cell cycles.

In Fig. 3 it is possible to observe adherent and floating cells stained by FITC-conjugated anti CD11a mAb (A, B) and FITC-conjugated isotypic control (B, C), respectively.

Floating cells showed a higher percentage of CD11a labelling than adherent cells. In fact, floating cells expressed a percentage of CD11a positivity varying from 24 to 60%, while adherent cells evidenced an LFA-1 percentage varying from 5 to 15%.

SK-N-MC cell morphology underwent relevant changes after hyperthermia (Fig. 4): detachment with concomitant cell rounding appeared (4A) and, within the suspended cell population, necrosis was frequently present (4B), showing the typical cytoplasmic hydration and membrane disruption. Apoptotic cells were also frequently recognizable among floating cells undergoing hyperthermia. Early apoptotic aspects (4C), with progressive chromatin margination, could be revealed, as well as late apoptotic features, with diffuse membrane blebbing, and apoptotic bodies (4D).

We performed five different experiments to evaluate both CD11a expression and supravital PI uptake in SK-N-MC cells, splitting the two subpopulations of floating and adherent cells before mAb labelling, as described in Materials and methods.

Cells that remained adherent did not seem to enter apoptosis, as shown by ultrastructural analysis and by flow cytometry, especially by means of supravital and fixed cell PI staining.

CD11a positivity, analysed on control and heat-exposed cells, is shown in Fig. 5A,C, while supravital PI uptake is shown in Fig. 5B,D. These data demonstrated that hyperthermia did not induce significant apoptosis, or a decrease in CD11a expression in SK-N-MC cells which remained adherent.

CD11a labelling and PI uptake on floating cells are represented in Fig. 5C,D, respectively. Significant differences ($p<0,05$) evaluated with Student's paired T test were found in both cases on control and hyperthermia-treated cells.

In Fig. 6 histograms and percentage values of a representative experiment are reported. FSC vs FL1 dot plots are shown from control (A, B) and hyperthermic (C, D) floating cells incubated with anti FITC-CD11a mAb (A, C) or FITC-isotypic control (B, D).

There was evidence that hyperthermia induced CD11a+ cell percentage decrease from 30% to 16%. FL1 vs FL3 dot plots (6E) show CD11a expression and PI uptake on control floating cells simultaneously. Normally, in this cellular fraction, a number of necrotic and apoptotic cells was found. Within PI+ SK-N-MC floating cells, 15% expressed CD11a and this molecule was also present in 10% of PI- non-apoptotic cells.

6F dot plot shows an identical determination performed on heat-treated cells. CD11a+/PI- non-apoptotic events appeared decreased to 5%, while CD11a+/PI+ cells to 10%. In the whole pool of floating cells, total PI+ events were 40% (30% CD11a-/PI+, 10% CD11a+/PI+), demonstrating that hyperthermia induced an increase of PI+ apoptotic cells.

CD11a expression was evaluated in two distinct subpopulations: dead (PI+ apoptotic/necrotic) and PI-

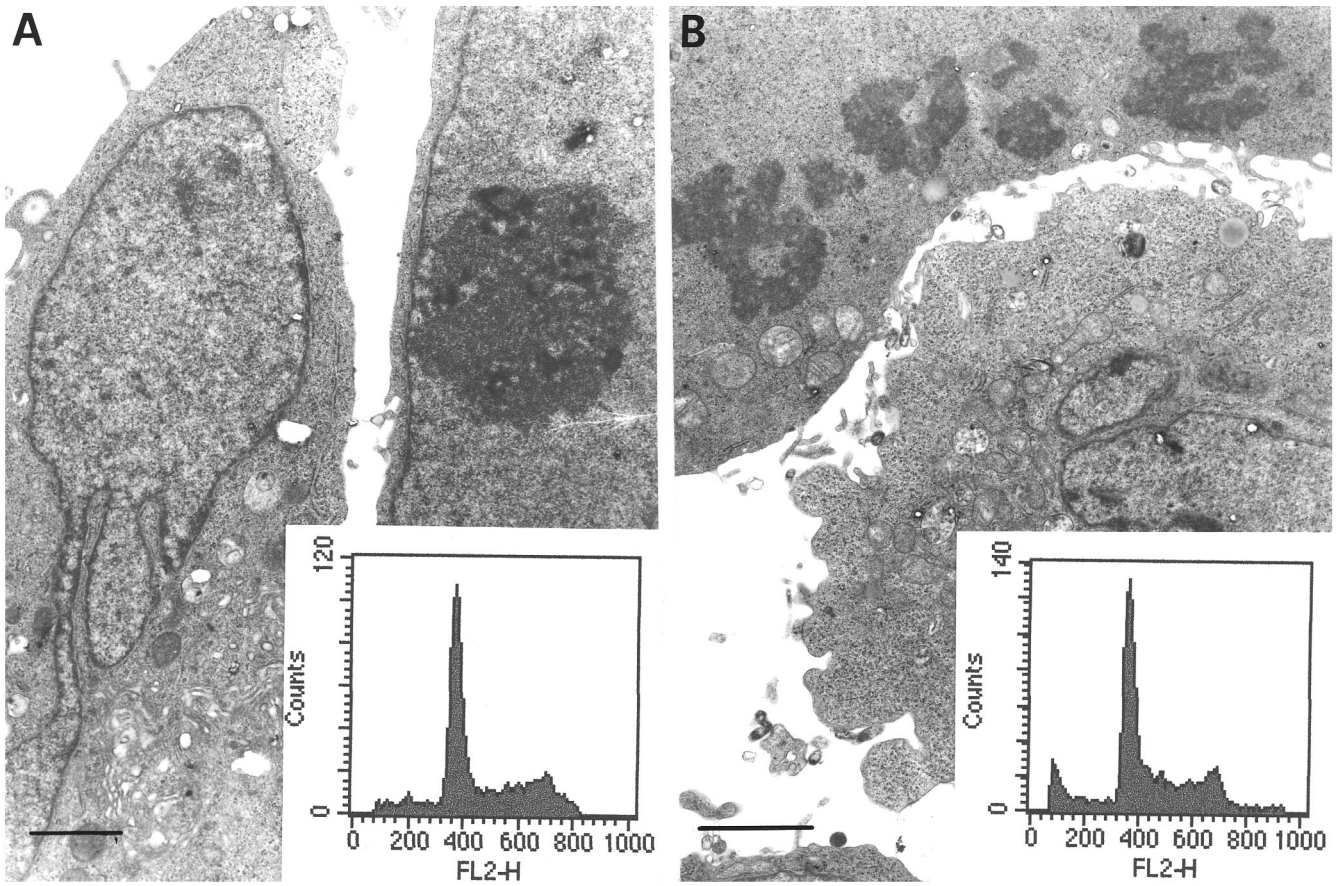


Fig. 2. TEM of adherent **(A)** and floating **(B)** SK-N-MC control cells: within the second population, together with rounded shape cells, mitoses can be found. The corresponding cell cycles appear in the insets. Bar: 1 μm.

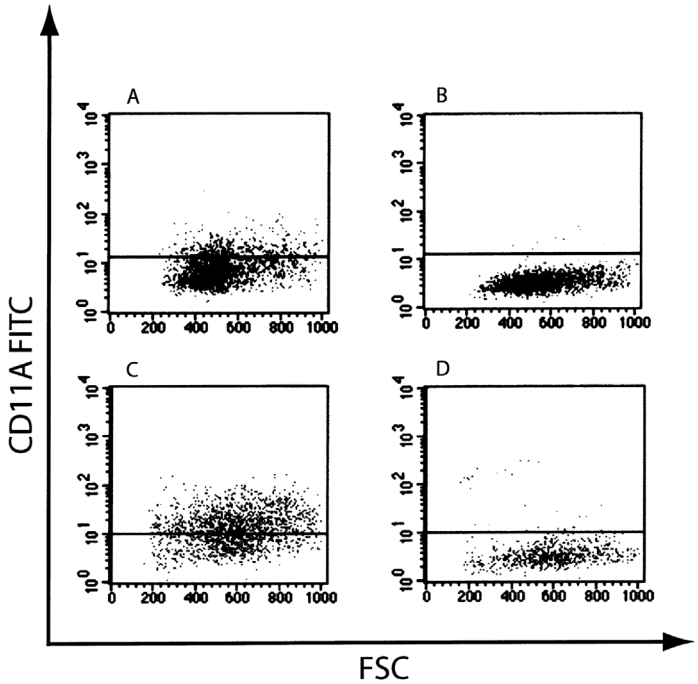


Fig. 3. Flow cytometry of adherent **(A, B)** and floating **(C, D)** control cells incubated with FITC-CD11a mAb **(A, C)** and FITC-isotypic control **(B, D)**: a higher positivity (58%) is shown by suspended cells.

viable cells. It was 55% in PI+ control floating cells, while such a percentage decreased to a value of 25% in PI+ treated floating cells.

Furthermore, in PI- control floating events, CD11a+ viable cells were 12.8%, while, after hyperthermic treatment, this percentage decreased to 6.6%.

This double analysis was repeated for each experiment (Fig. 7A,B). Data highlight a CD11a expression decrease in both PI^+ and PI- events, although this decrease appeared more evident in PI^+ than PI- cells.

SEM provided reliable information on cell shape (Fig. 8). In control condition (8A) most cells appeared adherent, with thin prolongations protruding towards the substrate and connecting cells to each other. Cell surface appeared characteristically covered with short microvilli. The floating population was less represented, but well recognizable by SEM too, as rounded regular cells. It strongly increased after hyperthermia (8B), which also

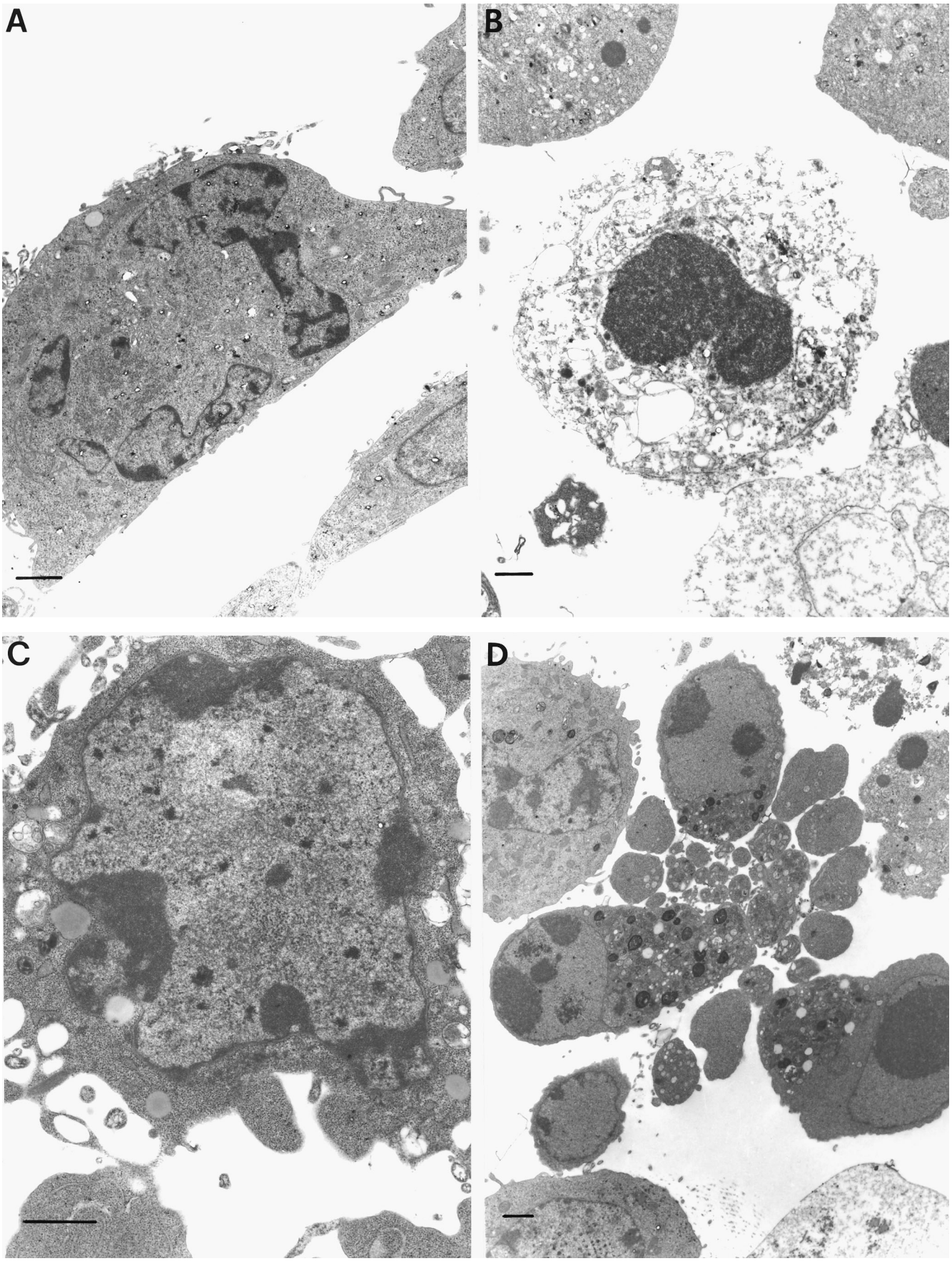


Fig. 4. TEM of SK-N-MC after hyperthermia: vs a scarcely represented adherent monolayer **(A)**, a growing floating population appears, including necrotic **(B)**, as well as early **(C)** and late **(D)** apoptotic cells. Bar: 1 μm.

determined a strong rearrangement of cell monolayer framework.

As evidenced by TEM, a variety of surface morphologies appeared in these samples. Microvillous coating could be present, but cells with a smooth surface also appeared (8C). Necrotic, widely destroyed cells (8D) could be also revealed, and diffusely blebbed ones - possibly apoptotic- could be found (8E).

Fig. 9 shows CD11a expression in SK-N-MC cells still floating after 24 h of adhesion.

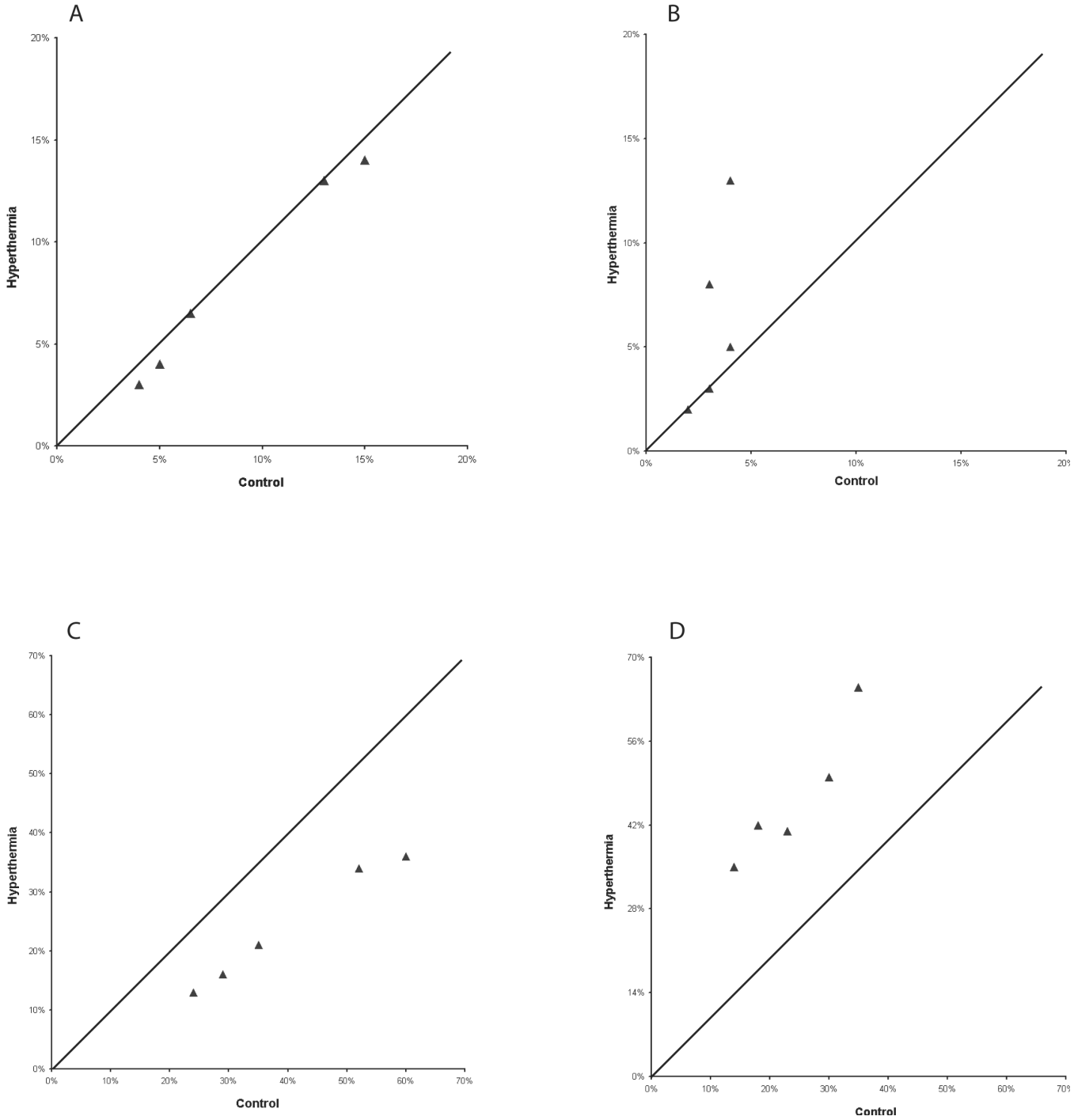
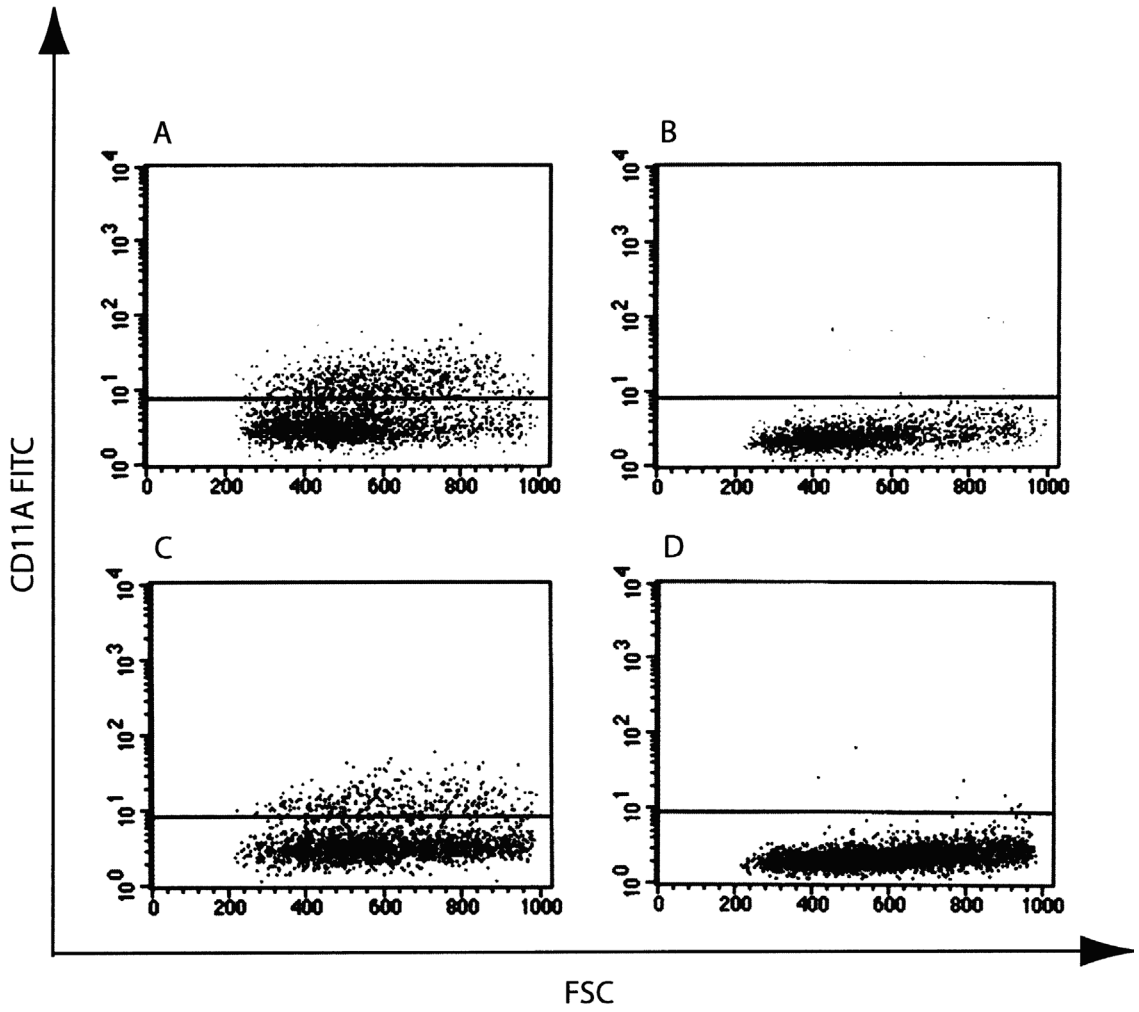


Fig. 5. CD11a positivity **(A,C)** and PI uptake **(B,D)** in control (x-axis) and hyperthermia-treated (y-axis) adherent **(A, B)** ($p>0,05$) and floating **(C, D)** ($p<0,05$) SK-N-MC cells. The line x=y is included as a reference in each panel.



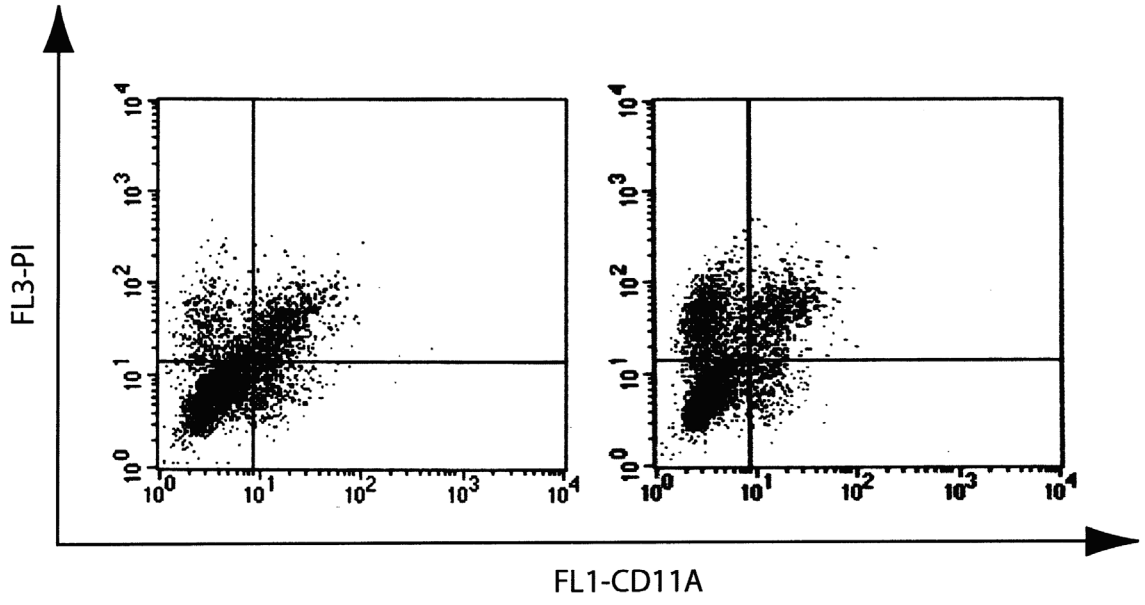


Fig. 6. Flow cytometry of untreated **(A, B)** and hyperthermic **(C, D)** floating cells incubated with FITC-CD11a mAb **(A, C)** and FITC-isotypic control **(B, D)**. FL1 vs FL3 dot plots for CD11a expression and PI uptake, from hyperthermic **(E)** and control **(F)** floating cells. Hyperthermia induces a CD11a expression decrease, as well as a simultaneous PI uptake.

FSC vs FL1 cytograms revealed that untreated floating cells (A) were CD11a+ in a 37%; on the other hand, floating cells (24 h after hyperthermic trigger) (B) showed a CD11a positivity decrease to 20%.

Fig. 9C-F shows DNA content of adherent cells derived from control (C, E) and hyperthermic (D, F) floating cells, after cell permeabilization and PI staining.

The adhesion test produced adherent cells from untreated and treated floating cells, showing different cell cycle patterns. Histograms 9E and F revealed necrotic events both in control and treated cells, although in the latter it was possible to identify a subdiploid peak between necrotic and G0/G1 events. In the first case, the percentage of necrotic events resulted in about 25% (9E), while apoptotic/necrotic events of adherent cells derived from treated floating ones was 60% (9F).

Histograms 9C and D revealed cell cycle phases referred only to vital cells.

Adherent cells from hyperthermic floating ones showed G2/M 16%, G0/G1 43% and S 40.5% events.

Adherent cells from controls showed G2/M 29.5%, G0/G1 43.5% and S 25% events.

Discussion

Tumor cells are characterized by uncontrolled growth, followed by migration and spreading to distant sites. Cancer mortality is frequently due to metastasis and surgical tumor removal can improve and prolong patient survival. Integrins are a family of transmembrane proteins composed of heterodimeric complexes of noncovalently linked α and ß chains, which mediate cell-cell and cell-ECM interactions and transduce signals from ECM to inner cell and viceversa .

Since these properties implicate their involvement in cell migration, as well as in intra- and extra-vasation, a role for integrins in tumor growth and metastatic diffusion is obvious.

On the other hand, apoptosis regulates the life span of normal cells and eliminates those undergoing toxic insults. Moreover, it takes part in cell detachment from ECM, generating, in this case, a peculiar type of death, also named “anoikis”

It is well known that hyperthermia is a powerful apoptotic inducer, which triggers a rapid death in a number, if not in all, of hemopoietic tumor cells (Falcieri et al., 2000a). In addition, its utilization in combination with chemo- and/or radiotherapy has been frequently practiced recently (Hildebrandt et al., 2002) and seems to represent a potentially important tool for cancer treatment (Van der Zee, 2002).

In this study we have demonstrate its action in SK-N-MC human neuroblastoma cells, which have been studied from a morphological and cytometrical point of view.

This particular model, consisting in an adherent and a floating double cell population, has been utilized to highlight the consequence of hyperthermia on cell viability and adhesion mechanisms, simultaneously.

The peculiarity of SK-N-MC line and its cell shape plasticity, widely evidenced by SEM, suggests an

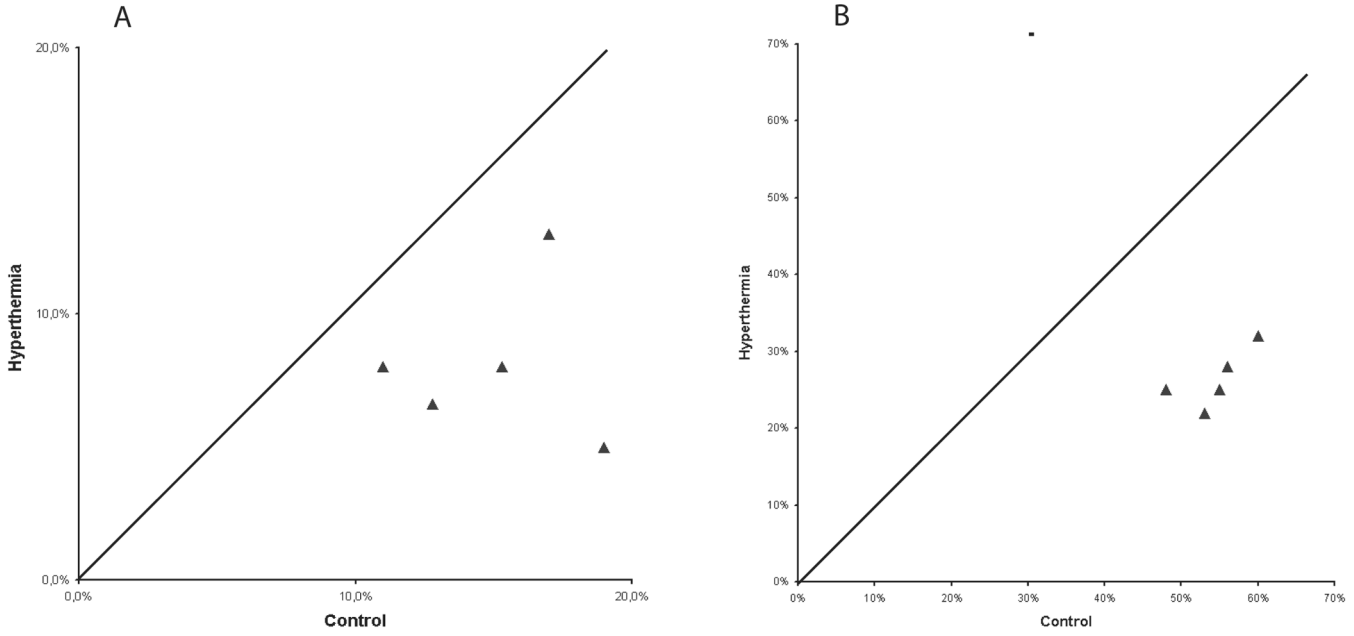


Fig. 7. Cross comparison of CD11a positivity related to PI+ (**A**) and PI- (**B**) events between control floating (x axis) and hyperthermia treated floating (y axis) SK-N-MC cells. The line x=y is included as a reference in each panel. Significant differences ($p<0,05$) evaluated with Student’s paired T test are found in both clusters.

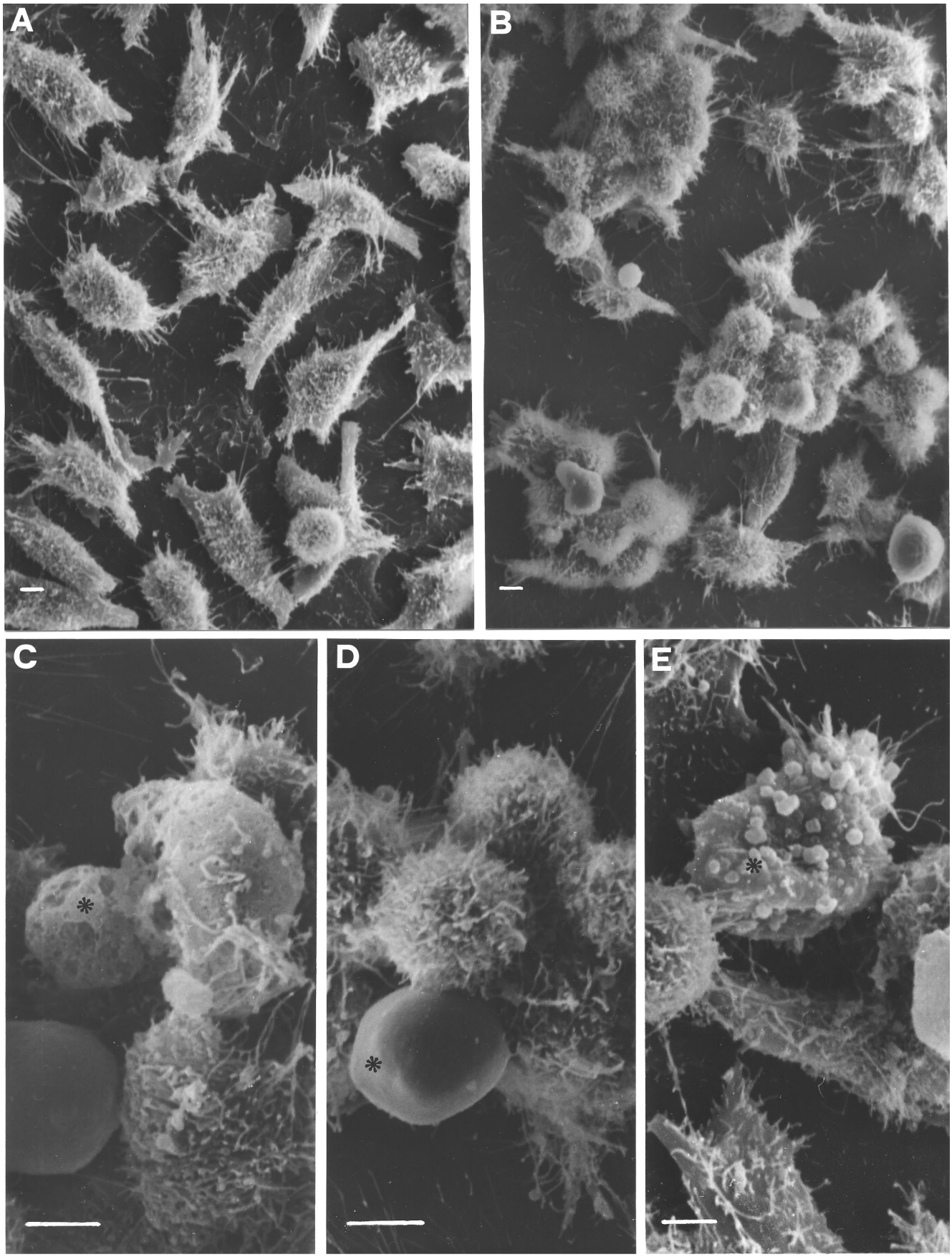


Fig. 8. SEM of SK-N-MC cells in control condition **(A)** and after hyperthermia **(B, C, D, E).** Loosening of cell monolayer and numerous rounded cells appear **(B)** after treatment, which show a smooth surface (**C**, *), a disrupted, presumably necrotic, cytoplasm (**D**, *) or a diffuse, possibly apoptotic, surface blebbing (**E**, *). Barr: 5 μm.

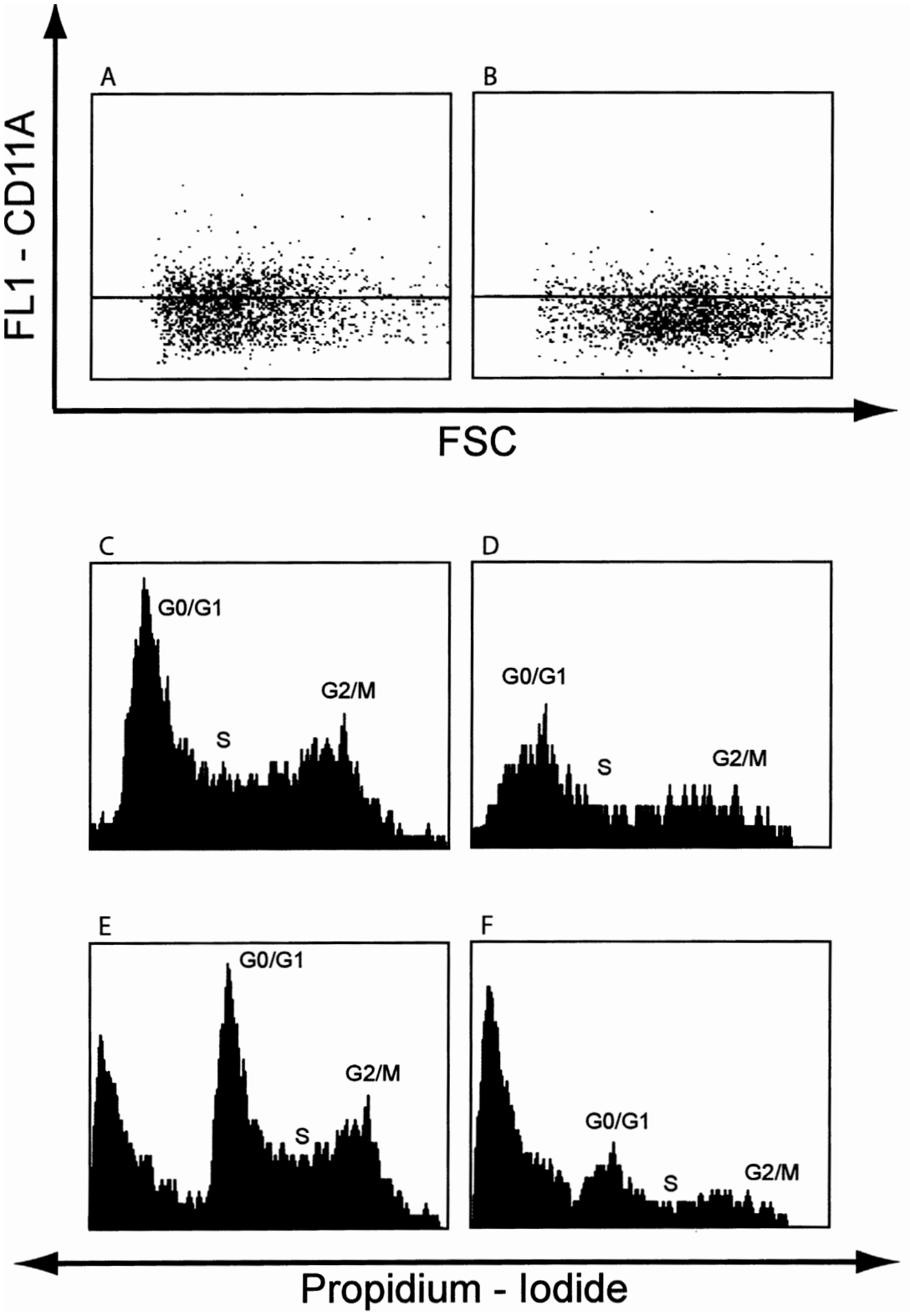


Fig. 9. Flow cytometry after 24 h re-adhesion test, applied to untreated **(A)** and hyperthermic **(B)** floating cells after FITC-CD11a incubation and their DNA content in the absence (**C, E)** and after hyperthermia **(D, F)** in the whole population **(E, F)** and limitedly to living cells (**C, D)**. The treatment gives a deep change in cell cycle phase distribution **(D)** and a sub-diploid peak **(F)**, identifying the apoptotic cell population.

important role of cytoskeleton in modulating cell shape changes and, particularly, membrane behavior. These considerations were the rationale for investigating neuroblastoma LFA-1, an adhesion molecule, which, even if diffusely described in leukocytes, appears one of the most relevantly cytoskeleton-correlated integrins (Brakebush et al., 2002).

CD11a immunolabeling in control cells showed a high positivity in suspended cells, while adherent ones were sensibly less reactive.

The most striking effect of hyperthermic treatment was cell monolayer detachment, which has been thoroughly analyzed. Mitotic, necrotic and, mostly, apoptotic cells could be revealed within specimens from floating cell pellets.

Moreover, hyperthermic treatment determined a CD11a expression decrease within suspended cells, particularly in the apoptotic ones, and, when the re-adhesion test was carried out in control and hyperthermic floating cells, a CD11a reduction again appeared in the latter. Hyperthermia also seems to affect cell adhesion capability.

It is licit to suppose that viable floating cells (G0/G1-S-G2/M cell cycle phases) derived from control specimens, are those involved in metastatic processes: this could be suggested, in this "in vitro" model, by the high percentage of CD11a positivity. Floating cells undergoing hyperthermia did not only show a significative increase in apoptotic and necrotic events, but also a decrease in CD11a expression. Even if a limited population was able to readhere, they showed strongly altered cell cycle phases, as suggested by flow cytometry of PI-stained fixed cells.

Taken together, our results suggest that hyperthermia can induce different effects: apoptosis and CD11a surface expression decrease on both apoptotic and living cells, even if it is less evident in the latter. These phenomena, interestingly, are not necessarily coupled.

Metastatic diffusion is thus affected by the presence of apoptotic cells and by adhesiveness impairment of living cells, both of which are consequent to hyperthermic treatment.

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