

## **Serum Factors Induced the Nuclear Location of Annexin V in the Human Osteosarcoma Cell Line (MG-63)**

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### **ABSTRACT**

**Calcium-binding proteins play essential roles in the cell. One important class of calcium-binding proteins is the annexin family. This is a family of 13 proteins, which binds to phospholipids in a calcium-dependent manner. Osteosarcoma cell line (MG-63) is a transformed cell that has many characteristics of the differentiated cell, such as a considerable serum dependency in its growth rate. Using specific antibodies against each annexin and immunofluorescence microscopy, the location and relocation of the annexin V was determined by some serum factors. Serum starvation of MG-63 cells increases their doubling time from 24 hours to 4 days. Cells grown in serum contain high levels of annexin V in the cell nucleus whereas in the absence of serum results in loss of nuclear annexin V in about 75% of the cells. Refeeding cells with medium containing 10% serum restore annexin V to the nuclei within 5 hours. Charcoal-treated serum cannot allow annexin V to return to the nucleus. Inhibition of protein synthesis with cycloheximide does not prevent the serum-induced return of annexin V to the nuclei. However, treatment of cells with genistein at a concentration specific for inhibition of tyrosine kinases (200 ~M) inhibits the relocation of annexin V from cytoplasm to the nucleus. Thus, the cellular location of the annexin V depends on the growth state of the cells. It can be altered by the movement of this protein between the cytosol and the nucleus. *Iran. Biomed. J. 4: 117-122, 2000***

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### **INTRODUCTION**

**A**n nexins are a family of calcium-binding proteins that have been identified in a wide variety of species and tissues. They are major cellular proteins with various intracellular and extracellular roles. [1, 2]. The possible intracellular role is modulation of mitogenic signal transduction [1, 2] and the extracellular roles include the inhibition of blood coagulation and the mediation of interaction between cell membranes and the extracellular matrix [1, 3]. Many growth stimulators, the cytosolic free calcium concentration and the division of some types of cells are prevented by inhibition of the calcium signal [1]. Both G-protein-linked and tyrosine kinase linked receptors can activate pathways leading to the generation of a calcium signal. The calcium can originate either from intracellular stores in the cytoplasmic reticulum or in the nuclear envelope [4, 5] or from

movement of extracellular calcium across the plasma membrane.

Annexins are the most common family of intracellular calcium-binding proteins that mediate the calcium signal. They contain a core domain of several repeating sequences, in highly conserved calcium-binding motif. In addition, annexins possess a less conserved N-terminal amino acid sequence that may be involved in targeting and regulating the annexin activities. Several members of the annexin family have been implicated in the regulation of growth processes [6].

Annexin V is one of the most widespread and abundant annexins. We have demonstrated that annexin V is expressed human osteosarcoma cell line MG-63 at high concentration in the nuclei of fibroblasts [7]. In this study, we investigated the effect of some serum factors on the tyrosine kinase signaling pathways in the nuclear location of annexin V.

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## MATERIALS AND METHODS

Tissue culture medium, enzymes, antibiotics, and fetal calf serum (FCS) were obtained from Gibco (UK). The polyclonal antiserum to annexin V was a kind gift from Dr. F. Seiler and Dr. J. Romisch (Behring, Marburg, Germany). Affinity-purified FITC-conjugated second antibodies, activated charcoal (Norit A), and cycloheximide were obtained from Sigma (UK). Genistein was purchased from calbiochem (UK).

MG-63 cells were cultured in 90-mm tissue culture dishes with Dulbecco's modified minimum essential medium (DMEM) containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 10 mg/ml streptomycin and were maintained at 37°C in 5% CO<sub>2</sub> and 95% air. Medium was changed after 4 days, and subculturing was performed every 7 days. Cells were released from the substratum by incubation with 0.05% trypsin and 0.02% (W/V) EDTA. After washing, the resulting suspension was diluted 1: 25.

**Proliferation rate of MG-63.** MG-63 cells were subcultured onto coverslips (1/25 dilution). After 3 days, they were refed with DMEM containing 10% FCS and after 3-4 days they were refed with DMEM containing 10%. Cells were counted by hemocytometer after 24, 48, 72 or 96 h.

**Immunofluorescence microscopy, and antiserum specificity.** MG-63 cells were subcultured on coverslips, and immunofluorescence was performed in culture after 3 to 7 days. After removal of the medium, cells were washed twice with PBS at 37°C and were fixed with neutral buffered formalin (Sigma, HT-50-1-128) at 37°C for 5 min. The cells were then permeabilized by incubation with 0.5% (V/V) Triton X-100 in PBS for 15 min at room temperature and refixed in formalin for 5 minutes. Subsequently, the cells were rinsed three times with PBS and incubated in 1 mg/ml NaBH<sub>4</sub> in PBS for 5 min. Non-specific binding sites were blocked by incubating the cells with PBS containing 5% (V/V) goat serum and mM sodium azide at room temperature for 20 min, and then the cells were incubated with antibodies against human annexin V for 3 h. Antisera were centrifuged for 5 min at 12,000 ×g and then diluted 1:100 with PBS containing 5% (V/V) goat serum and 1mM sodium azide. After incubation with first antibody, the slides were washed eight times with PBS and then incubated for 3 h with FITC-conjugated anti-rabbit IgG diluted 1:100 in PBS containing 5% (V/V) goat

serum and 1 mM sodium azide. Then slides were washed eight times with PBS, mounted in Vectashield mounting medium, and viewed with a Nikon optiphot fluorescence microscope. Preincubation of the antiserum to annexin V with pure annexin V was able to totally remove immune staining obtained with the polyclonal antiserum. The anti-serum to annexin V has been previously shown to be specific by Western blotting analysis of several cell types including MG-63 cells [7, 8].

**Preparation of charcoal-stripped FCS (CS-FCS).** In some experiments, FCS was depleted of low-molecular-weight peptides and metabolites containing a steroid-ring structure by treatment with activated charcoal. Norit A (40 mg/ml) was added to FCS and the resulting suspension incubated with mixing at 4°C for 90 min. The mixture was then centrifuged at 8,000 ×g for 40 min at 4°C. The supernatant containing FCS was mixed with fresh charcoal and the incubation and centrifugation steps were repeated. The supernatant was then sterilized by passing it through a 0.2 μm filter.

**Inhibition of protein synthesis.** Cells were depleted of nuclear annexin V by removal of serum from culture medium for 4-5 days. Medium was then replaced with DMEM containing 10% (V/V) FCS plus 100 μg/ml cycloheximide to inhibit protein synthesis. After 5 and 24 h, annexin V was detected by immunofluorescence, as previously described.

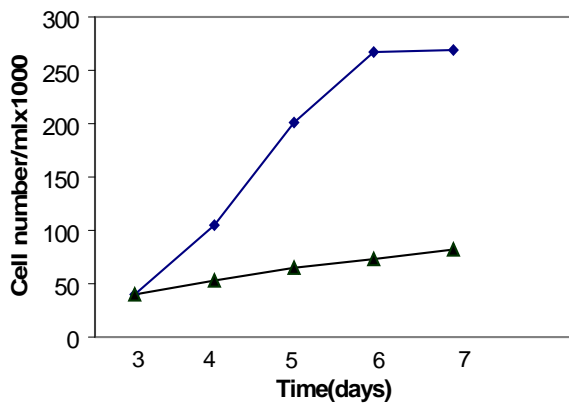
**Effect of depletion of FCS from the culture medium or its replacement with CS-FCS.** After 3-4 days in culture, cells were rinsed with serum-free DMEM and then were incubated with DMEM supplemented with 10% (V/V) FCS or with 10% (V/V) CS-FCS for up to 96 h. In other experiments, the effect of reintroducing CS-FCS in the medium that were previously depleted of nuclear annexin V was examined by first incubating the cells for 96 h in serum-free medium and then by replacing with DMEM containing 10% (V/V) CS-FCS and culturing the cells for a further 24 h.

**Inhibition of tyrosine kinase activity.** After 3-4 days in culture, cells were rinsed with PBS and incubated with serum-free DMEM (0.1% W/V BSA) for 4-5 days. They were then refed with 10% (V/V) FCS containing 25-200 μM genistein and

incubated for further 24 h, after which annexin V was detected by immunofluorescence microscopy.

## RESULTS

**Nuclear location of annexin V depends upon growth conditions.** MG-63 cells were subcultured (1:25 dilution), refed on day 3 and then incubated for further 3-4 days after passaging cells were detached and counted with a hemocytometer. MG-63 cells proliferated rapidly up to day 6 (Fig. 1) with a doubling time of 24 hours. In the absence of serum, MG-63 cells continued to grow slowly with doubling time of 4 days (Fig. 1).



**Fig. 1:** Proliferation of MG-63 cells cultured in the presence and the absence of FCS. Cells were seeded into 6 well plates and refed on day 3 with medium containing 10% FCS (○) or without FCS (△). Cells were detached using trypsin (0.05% W/V) EDTA (0.02% w/v) and were counted using hemocytometer. Results are presented as the mean ± SE of triplicate experiments.

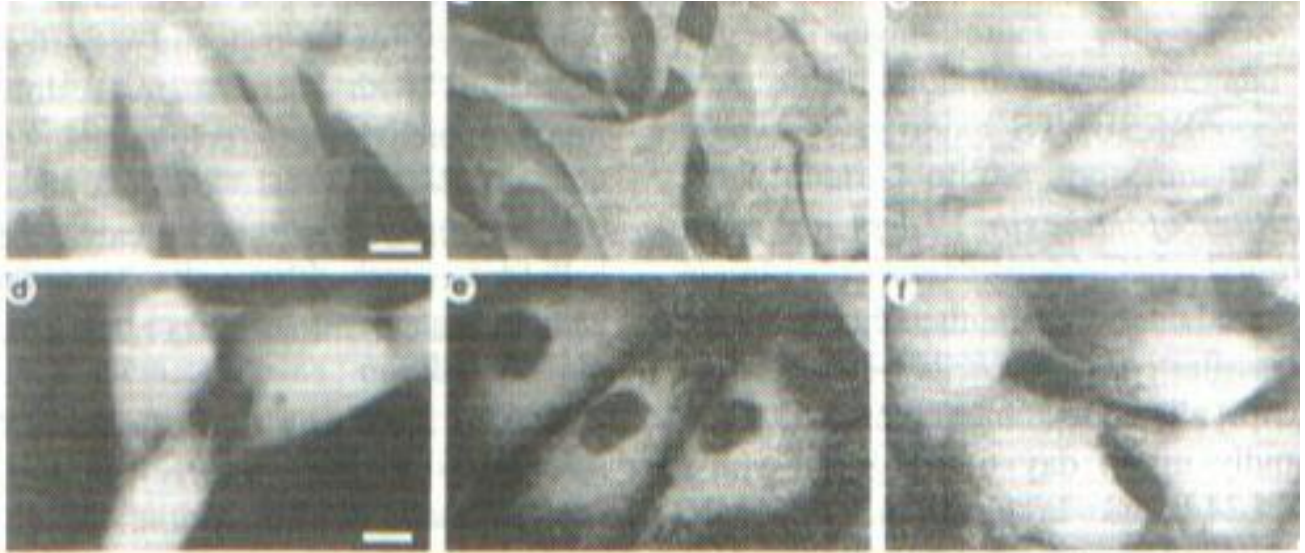
In non-confluent cells (3-5 days after passage), annexin V was present both in the cytoplasm and the nucleus of most cells. MG-63 cells were refed three days after passaging and incubated for further 4-7 days prior to performing immunocytochemistry. Under these conditions, annexin V was absent from the nucleus of most cells (Fig. 2). Refeeding these cells with fresh medium containing 10% serum restores nuclear annexin V within 5 or 24 hours (Fig. 2c).

Loss of annexin V from MG-63 cell nuclei can also result from growing cells in the absence of serum (Fig. 2e). Thus, after culturing the cells 4 days in the absence of serum,  $72 \pm 4\%$  of cells

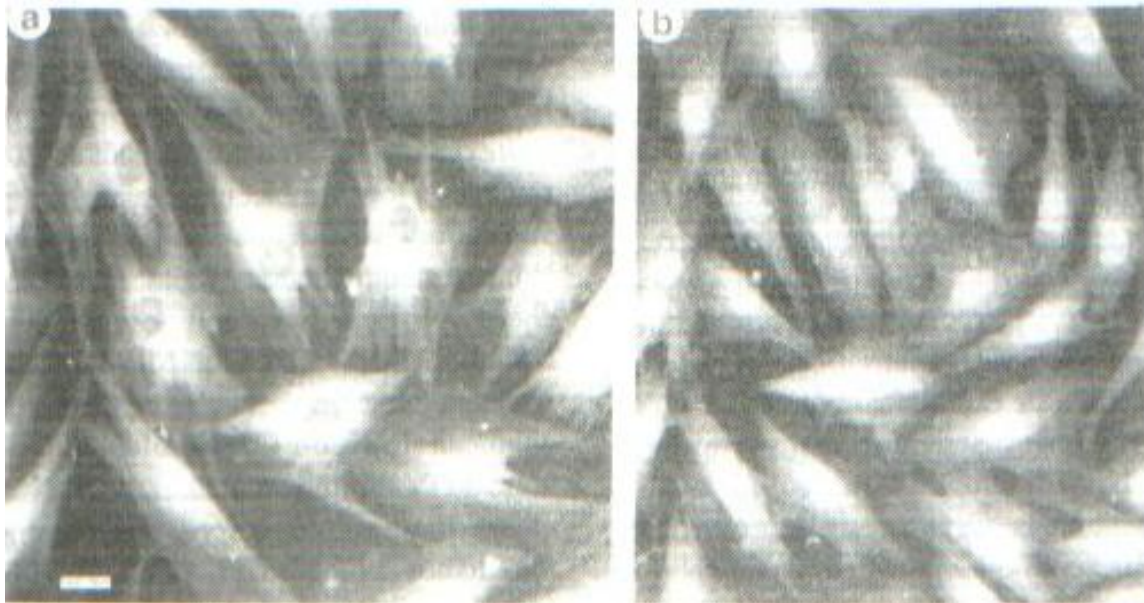
have lost nuclear annexin V (Fig. 2e). Refeeding the cells with medium containing 10% serum caused the return of nuclear annexin V in more than 95% of the cells within 24 h (Fig. 2f). The degree of confluency of the cells did not affect the ability of refeeding with serum to restore annexin V immunoreactivity to cell nuclei. Quantitation of annexin V in total cell extracted by Western blotting showed no significant differences in the cellular content of annexin V under the different cell culture conditions (data not shown).

**Protein synthesis is not required for annexin V relocation to the nuclei on refeeding serum-starved cells with serum.** Cycloheximide is a substance that can be used to inhibit protein synthesis in MG-63 cell [9]. MG-63 cells were starved of serum to induce the loss of nuclear annexin V (Fig. 3a). Subsequently, cells were refed together with cycloheximide at concentrations known to inhibit protein synthesis. Despite the presence of cycloheximide, annexin V relocated to the nuclei within 5 h (Fig. 3b). These results clearly demonstrate that protein synthesis is not involved in the relocation of annexin V to nuclei (Fig. 3b).

**Charcoal treatment of FCS removes factors responsible for the nuclear relocation of annexin V.** Charcoal treatment of FCS is known to remove low molecular weight peptides and substances with a steroid-ring structure. Therefore We tested whether replacement of FCS with charcoal-stripped caused loss of annexin V from nuclei. Also, refeeding MG-63 cells depleted of nuclear annexin V with medium containing 10% charcoal-stripped-FCS resulted in the return of annexin V to the nucleus. FCS was treated with charcoal under conditions described previously for the removal of steroid hormones and peptide growth factors. Cells were grown for 4 days in culture without serum, at that time 25% of cells contained nuclear annexin V. Cells were then either refed with charcoal-stripped or with untreated FCS. Cells refed with untreated FCS showed a recovery of nuclear annexin V in about 88% of the cells after 24 h, whereas, there was no significant change in the extent of nuclear annexin V in cells treated with charcoal stripped serum.



**Fig. 2.** Location of annexin V in MG-63 cells. MG-63 cells were subcultured in DMEM containing 10% FCS and refed after 3 days. Immunofluorescence was performed on: (a, d) cells 3 days after passaging, (b) confluent cells 9 days after passaging without a further change of medium and (c) confluent cells as in (b) 24 h after refeding with DMEM containing 10% FCS. In (e), cells were passaged and allowed to grow for 3 days in DMEM containing 10% FCS. On the third day, cells were washed and refed with medium containing no FCS and maintained for further 3 days. In (f), cells were treated as in (e), then refed with medium containing 10% FCS and maintained for a further 24 h. Scale bars in (a) and (d) are representative for all panels of the figure and indicate 10  $\mu$ m.



**Fig. 3.** Inhibition of protein synthesis does not prevent serum-induced relocation of annexin V to nuclei. Cells were passaged and allowed to grow for 3 days in DMEM containing 10% FCS. On the third day, cells were washed and refed with medium containing no FCS, maintained for a further 4 days, and then refed with fresh medium containing 10% FCS and 100  $\mu$ g/ml cycloheximide. After a further 5 h, immunofluorescence microscopy was performed. The location of annexin V is shown (a) after removal of FCS from the culture medium for 4 days and (b) after removal of FCS from the culture medium for 4 days and then refeding with medium containing FCS and Cycloheximide. The scale bar (-) represents the magnification of 10  $\mu$ m in (a) and (b).



**Fig. 4.** Tyrosine kinase inhibition attenuates the return of annexin V to the nucleus. MG-63 cells were subcultured in DMEM containing 10% FCS and maintained for 3 days. On day 3 they were washed and refed with serum-free medium in which they were maintained for a further 4 days. They were then refed fresh medium containing 10% FCS+100 $\mu$ M genistein. Immunofluorescence was performed after 24 h. The location of annexin V is shown (a) after 4 days in serum-free medium, (b) after refeding with medium containing 10% FCS and 100 $\mu$ M genistein and (c) after refeding with medium containing 10% FCS without genistein.

***Inhibition of tyrosine kinases blocks the return of annexin V to the nuclei of serum-starved cells.***

To assess the involvement of tyrosine kinase pathway in the reappearance of annexin V in the nuclei, the protein kinase inhibitor, was used. There was little effect of 25 and 50  $\mu$ M genistein on intact cells (data not shown), whereas 100 and 200  $\mu$ M genistein showed a pronounced effect. Figure 4 shows the effect of 100  $\mu$ M genistein to serum-starved cells together with FCS. It is clear that genistein considerably inhibited the return of annexin V to nuclei. Nuclear staining for annexin V was much lower than cytoplasmic staining (Fig. 4b vs Fig. 4c).

## DISCUSSION

Previous immunohistochemical studies have shown that annexin V is present at an elevated concentration in the nuclei of cells in the culture [6, 10]. Its location is consistent with the association of annexin V with the nuclear matrix [9]. The intranuclear location of annexin V has been previously shown to resemble that of nuclear matrix components of confocal microscopy [10] and by biochemical extraction of cells prior to immunofluorescence microscopy. The size of annexin V should allow it to diffuse freely between the nucleus and the cytoplasm but the sequence of annexin V does not contain a nuclear location signal homologous to those found in the SV-40 large T antigen or in nucleoplasm [11]. Thus, the elevated level of annexin V in the nucleus

compared to cytoplasm argues for its interaction with nuclear components, as is seen for other nuclear proteins such as nucleotin [12].

We tested the reappearance of annexin V in the nucleus by 100  $\mu$ g/ml cycloheximide to block protein synthesis of cells [11]. This experiment has two important consequences: first, it indicates that there are not distinct forms of annexin V varying in sequence and with different locations in the cell; second, it indicates that the relocation of annexin V is not due to the synthesis of a specific binding protein that translocates to the nucleus. Instead, this result strongly suggests that the relocation of annexin V to nuclei is a consequence of a signaling pathway initiated by serum factors and resulting in the modification of annexin V or annexin V-binding components. There is no evidence for post-translational either modification of annexin V or its phosphorylation [3, 13]. In stimulated cells, we have seen electrophoretic mobility that would be likely to occur on post-translational modification [8].

FCS contains vital factors that support survival of many mammalian cells in culture, including stimulator of cell growth, attachment and spreading factors. Many of these agents control cell proliferation by binding to cell surface receptors. FCS was treated with charcoal under conditions described previously for the removal of steroid hormones and peptide growth factors [5]. These results argue strongly for an involvement of serum factors in mediating the nuclear relocation of annexin V. The involvement of steroid hormones seems unlikely since 5-h treatment with

cycloheximide does not block the return of annexin V to nuclei, whereas protein synthesis is an important element in the action of steroid hormones. Therefore, it is more likely that peptide hormones are involved in the signaling pathways that mediate the relocation of annexin V to nuclei.

Several serum-derived factors are mitogenic for osteoblast-like cells including EGF, IGF [13, 14]. In particular, TGF- and IGF-1 have all been shown to stimulate division osteoblast-like cell lines after serum starvation. Many of these peptide growth factors interact with cell surface receptors with tyrosine kinase activities that signal to the nucleus via pathways involving MAP kinase [9, 15]. The protein kinase inhibitor genistein attenuated the relocation of annexin V to the nucleus implicating one or more tyrosine kinase activities in this process. At the concentration used (100  $\mu$ M), genistein is claimed to be specific for tyrosine kinases. IC<sub>50</sub> values of genistein for tyrosine kinases varies between 2.5 and 25  $\mu$ M, consequently, 100  $\mu$ M genistein should preferentially inhibit tyrosine kinase activity [11, 16]. Figure 4 shows the effect of adding 100  $\mu$ M genistein to serum-starved cells together with FCS. This shows clearly that genistein considerably inhibits the return of annexin V to nuclei with nuclear staining for annexin V being much lower than cytoplasmic staining.

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