

Antisense EGFR sequence enhances apoptosis in a human hepatoma cell line BEL-7404

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ABSTRACT

Effects of antisense epidermal growth factor receptor (EGFR) sequence on apoptotic cell death were examined in a human hepatoma cell line BEL-7404 cells. In the cells of JX-1, a sub clone of BEL-7404 stably transfected with antisense EGFR vector (Cell Research, 3:75, 1993), an enhanced rate (9.5%) of spontaneous apoptosis was detected by flow cytometry, whereas the rates of spontaneous apoptosis in JX-0 cells, a sub-clone of BEL-7404 transfected by control vector, and the parent BEL-7404 cells were almost equal and about 1.7%. Serum-starvation for 72 h increased the rate of apoptosis of JX-1 cells up to 33.7%, while JX-0 and BEL-7404 cells, under the same condition, produced less than 5% of apoptotic cells. Observation with electron microscope demonstrated that condensation and fragmentation of chromatin and formation of apoptotic bodies often occurred in JX-1 cells, especially during serum-starvation. These results, combined with the data of DNA fragmentation Elisa test, suggested that antisense EGFR sequence enhances apoptosis in the human hepatoma cells.

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Comparison of intracellular Ca^{2+} level and the responsiveness of JX-1 cells to the induced action of EGF and thapsigargin (TG) treatment with that of control JX-0 cells indicated that antisense EGFR might interrupt the EGF/EGFR signaling pathway resulting in the decrease of intracellular Ca^{2+} pool content as well as the responsiveness of these cells to the extracellular signals. These findings suggest that antisense EGFR either directly or indirectly regulates Ca^{2+} storage in endoplasmic reticulum, thereby enhances apoptosis in the human hepatoma cells.

Key words: *Antisense EGFR, hepatoma cells, apoptosis.*

INTRODUCTION

Apoptosis is an active form of programmed cell death different from necrosis. Like cell proliferation and differentiation, apoptosis is a characteristic event or process which is regulated by both intracellular and extracellular signals[1-3]. Recently, the increasing evidence implies that alteration in apoptosis contribute to tumorigenesis and thus treatment designed to alter apoptosis may have the potential to change the progression of cancer[4].

The epidermal growth factor receptor (EGFR) is a 170 KD glycoprotein which contains essentially an extracellular binding domain, a transmembrane domain and an intracellular protein kinase domain. The EGFR tyrosine kinase-mediated signal transduction pathways have been considered to regulate many cellular processes. Amplification and overexpression of the EGFR has been detected in a number of human tumors of epithelial origin[5]. The sequence homology has been found between intracellular portion of the EGFR and $\text{gp}^{68\text{v-erbB}}$, the transforming protein of oncogene v-erbB [6]. The EGFR activation has been implicated in autocrine stimulation of cell growth in many experimental system[7]. Therefore, the EGFR might be responsible for the genesis and the maintenance of malignant properties of certain types of tumors and it appears to be a good target for antitumor therapy.

In the previous studies, we have demonstrated the EGFR gene expression and mitogenic effects of EGF in cells of a human hepatoma cell line BEL-7404[8]. Furthermore, we have obtained an antisense EGFR vector transfected BEL-7404 cell clone, termed JX-1 cells, showing amazing alteration in growth behaviors[9]. We describe here that antisense EGFR sequence enhances apoptotic cell death in the transfected JX-1 cells and its underlying mechanism will be discussed.

MATERIALS AND METHODS

Materials

Fluo-3/AM, propidium iodide and thapsigargin were purchased from Sigma (St. Louis, MO, USA). EGF was the product from Gibco BRL (Gaithersburg, MD, USA).

Cell culture

Human hepatoma cell line BEL-7404 was established in our Institute. BEL-7404 cells and the derivatives were grown in Dulbecco's modified Eagles's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 13% fetal calf serum, 100 u/ml penicillin and 100 mg/ml streptomycin in 5% CO₂ incubator at 37°C and cultured medium was changed every 48 h.

Stable transfectants

The recombinant plasmid containing antisense EGFR sequence was co-transferred with pSV2-neo into BEL-7404 cells by electroporation, followed by selection and cloning in the presence of G418 (400 µg/ml). The parent vector plasmid pEV-1 was used as control for transfection. Two representative cell lines JX-1 transfected with antisense EGFR sequence and JX-0 transfected with control vector pEV-1 were cloned for further analysis.

Electron microscopy

For transmission electron microscopy analysis, exponentially growing JX-1 cell in monolayer culture were collected by trypsinizing. The cells were fixed in 4% glutaraldehyde, postfixed in 1% OsO₄, and embedded with Epon618. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and viewed in a JEWI00B electron microscope.

Flow cytometry

The cells grown in monolayer culture were trypsinized and centrifuged at 250×g for 5 min. The cell pellet was resuspended in 1 ml hypotonic fluorochrome solution (propidium iodide 50 µg/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma). The sample was kept at 4°C in dark overnight. The propidium iodide fluorescence of individual cells was measured by using a FACStar Plus flow cytometry (Becton and Dickson). The cells with subdiploid DNA content were considered as apoptotic cells. Each value represented the mean of 10,000 cells.

DNA fragmentation Elisa assay

Cellular DNA fragmentation was measured by using a commercial available DNA fragmentation Elisa kit (Boehringer Mannheim) which is based on the quantitative sandwich enzyme immunoassay principle using two mouse monoclonal antibodies directed against DNA and BrdU respectively. This allows the specific detection and quantification of Brdu-labeled DNA fragments. Briefly, the cells (1×10⁴) were seeded to 96-well flat bottom cell culture plate (Corning). Exponentially growing cells were incubated with 10 µM BrdU for 12 h in 5% CO₂ incubator at 37°C and then lysed by adding lysis buffer. After lysis, the samples were centrifuged at 250×g for 10 min and 100 µl of supernatant removed from each sample for Elisa assay. The samples were added to microtiter plate precoated with anti-DNA antibody and incubated for 90 min. After the samples were incubated with anti-BrdU peroxidase for another 90 min, TMB substrate was added to plate. After incubation in the dark for 20 min, the reaction was terminated by adding 25 µl of 1M H₂SO₄ to each well. Measurement of absorbance at 450 nm was performed using microplate reader (Bio-Rad).

Measurement of intracellular free Ca²⁺ concentration

Cytosolic Ca²⁺ concentration was measured by fluorometry of furo-2-loaded or fluo-3-loaded cells as described[18].

RESULTS AND DISCUSSION

EGF/EGFR pathway is known as an important regulating network for the growth control in either normal cells or malignant cells. We reported here the study of the role of EGFR in apoptosis of human hepatoma cells using antisense EGFR vector transfectants as a model. Flow cytometry combined with propidium iodide-staining showed that an increased number of sub-G1 phase cells, which contained subdiploid DNA, occurred in antisense EGFR transfected cell line JX-1 cells in comparison to those in control vector transfectants JX-0 cells and the parent BEL-7404 cells (Tab 1, left column). The percentage of cells with subdiploid DNA content in JX-1 cells was 9.5%,

Tab 1. The effects of serum starvation on apoptosis of BEL-7404, JX-0 and JX-1 cells*

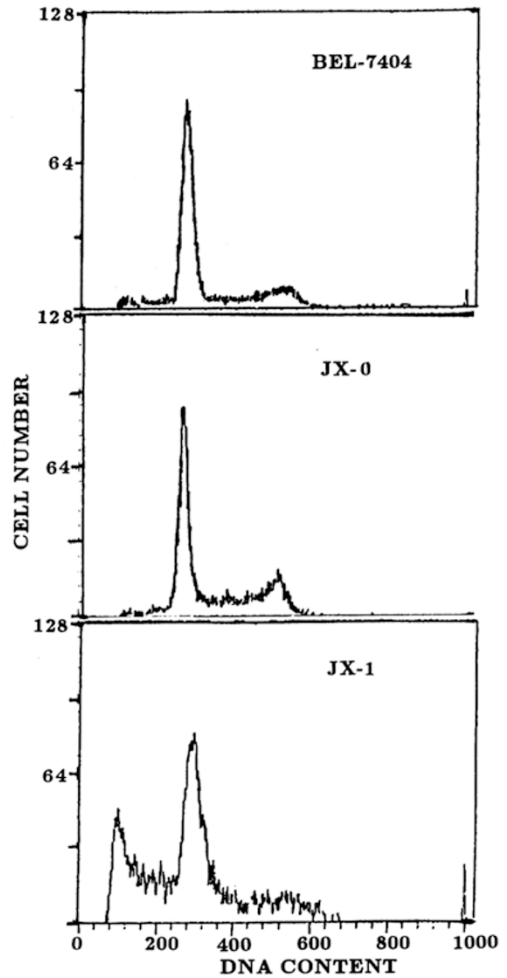
Cells lines	% cells with subdiploid DNA content (M±S.D.,n=3)	
	Serum (+)	Serum (-)
Bel-7404	1.65±0.24	2.28±0.43
JX-0	1.72±0.15	3.25±0.38
JX-1	9.51±1.69	18.41±1.03

* 80% confluent cells of BEL-7404, JX-0 and JX-1 were cultured in the fresh DMEM with or without 13% FCS for 24 h before harvesting.

whereas those in JX-0 and BEL-7404 cells were similar and about 1.7%, indicating an enhanced rate of spontaneous apoptotic cell death in JX-1 cells. In the previous study[9], we have demonstrated that BEL-7404 and control vector transfectants JX-0 cells can grow in serum-free medium, while JX-1 cells can not survive during the serum-starvation. In the present study, it was found that after serum-starvation for 24 h, the rate of apoptosis of JX-1 cells was increased up to 18.4%, but JX-0 and BEL-7404 cells only showed less than 2-3% apoptotic cells (Tab 1, right column). When the serum-starvation was elongated to 72 h, as shown in Fig 1, the rate of apoptotic cells with subdiploid DNA content were increased up to 33.7% in JX-1 cells, but less than 5% apoptotic cells were found in JX-0 and BEL-7404 cells. Data obtained with DNA fragmentation Elisa test also supported that JX-1 cells had a higher rate of apoptotic cell death than the control vector transfectants, especially during serum-starvation (Tab 2). Ultrastructure analysis of JX-1 cells demonstrated the occurrence of some characteristic morphologic changes of apoptosis, including condensation and fragmentation of chromatin and formation of apoptotic bodies (Fig 2).

Effects of EGFR on apoptosis is steadily gaining attention in recent years[10-13]. EGF can maintain the survival of the mouse embryo cell under serum-free condition, while deprivation of EGF induces these cells undergoing apoptosis[10]. An anti-EGFR monoclonal antibody has been reported to block activation of EGFR

Fig 1. Flow cytometric DNA content analysis of BEL-7404, JX-0 and JX-1 cells. These cells were grown in the absence of serum for 72 h before harvesting. X-axis represents fluorescence intensity and Y-axis represents cell members.



Tab 2. Effects of serum-deprivation on DNA fragmentation in JX-0 and JX-1 cells*

Serum-free duration (h)	DNA Fragmentation (OD, $M \pm S.D.$, n=4)	
	JX-0	JX-1
0	0.163 ± 0.047	0.456 ± 0.172
24	0.305 ± 0.062	0.956 ± 0.117
48	0.278 ± 0.064	1.134 ± 0.207

DNA fragmentation was measured by using the DNA fragmentation Elisa Kit.

tyrosine kinase and induce apoptosis in some human colorectal carcinoma cells[11]. We also observed that the monoclonal antibody against EGFR obviously inhibited the growth and induced the apoptosis of BEL-7404 cells *in vitro* (unpublished data). However, Armstrong et al. have found that activation of EGFR induces apoptosis in some human breast cancer cells[13]. These discrepancy implies that the

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EGFR-mediated multiple signal transduction pathways may have different effects on apoptosis.

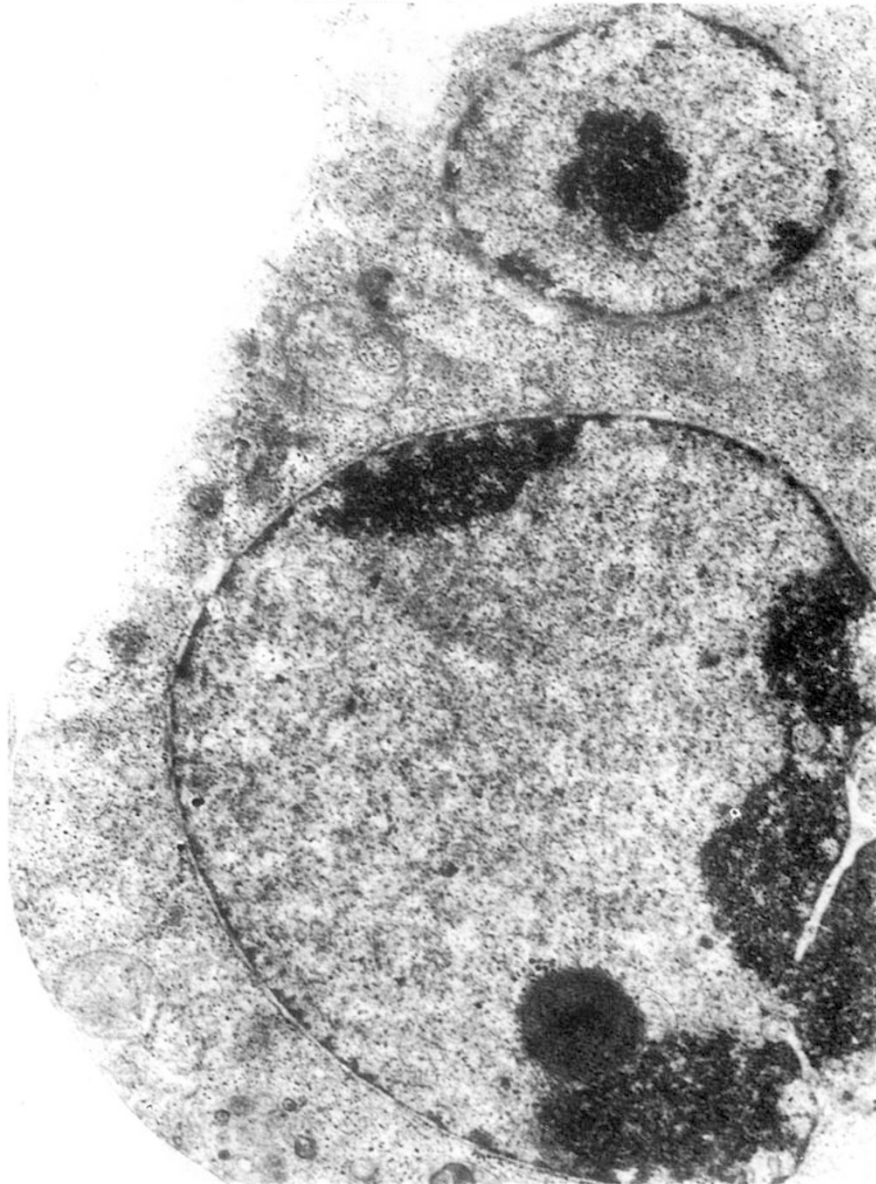


Fig 2. Ultrastructure of JX-1 cells in the absence of serum for 48 h, observed by transmission electron microscope (x 6,000)

How does EGFR regulate the growth and apoptosis in human hepatoma cells? EGF binds its receptor and stimulates a series of rapid response including phosphorylation of tyrosine residues within the EGFR itself and many other cellular proteins, hydrolysis of phosphatidyl inosital, release of Ca^{2+} from intracellular stores and morphological changes. EGFR plays a key role mediating the signal transduction. The deregulation of EGFR expression and function is supposed to disturb the signal pathways in the cells.

As a member of second messengers, calcium may be a sensitive target responding to the modulation of EGFR activities. We have found that TG, an inhibitor of Ca^{2+} - ATPase on surface of endoplasmic reticulum induces a transient increase of intracellular Ca^{2+} in BEL-7404 cells and disturbs the response of intracellular

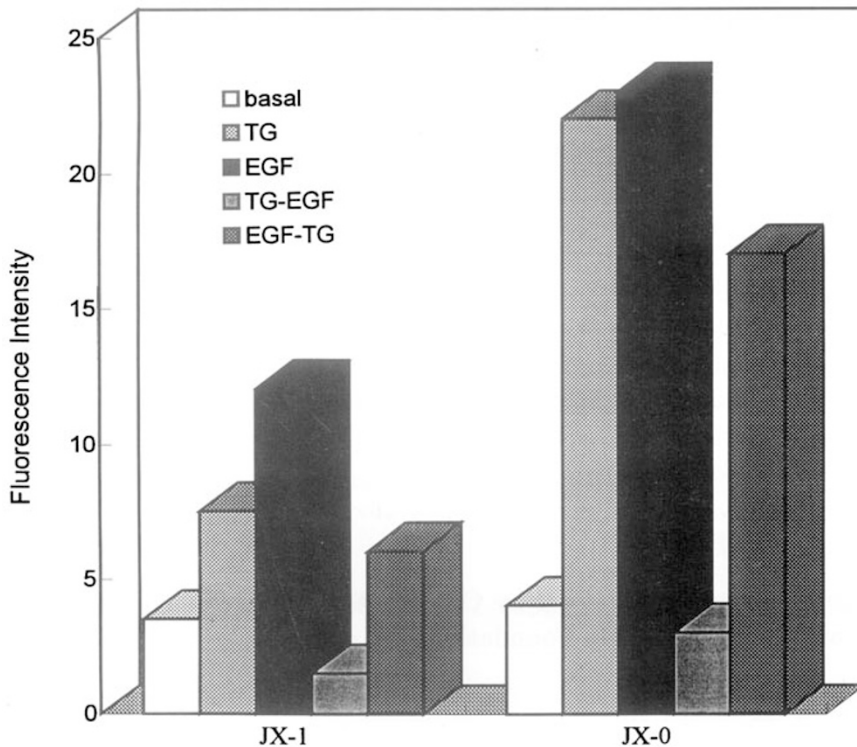


Fig 3. Effects of TG and EGF on intracellular Ca^{2+} level in JX-0 and JX-1 cells. Intracellular free Ca^{2+} levels were measured in fluo-3-loaded cell populations as described in ref. 14. The peaks were recorded after the application of either EGF (300 ng/ml) or TG (100 nM) for 1 min. EGF-TG: after the pre-incubation with EGF (300 ng/ml) for 10 min, without washing, TG (100 nM) was added for 1 min before intracellular Ca^{2+} measurement. TG-EGF: after the pre-incubation with TG (100 nM) for 20 min, without washing, EGF (300 ng/ml) was added for 1 min before intracellular Ca^{2+} measurement.

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Ca²⁺ following the stimulation with EGF, due to the depletion of the intracellular Ca²⁺ storage in endoplasmic reticulum[14, 15]. In this study, we compared the intracellular Ca²⁺ level and response to the induction of EGF and TG treatments in antisense EGFR transfected JX-1 cells and control vector transfected JX-0 cells. As shown in Fig 3, short-term treatment of 100 nM TG or 300 ng/ml EGF induced the release of Ca²⁺ in both JX-1 and JX-0 cells. The response was much weaker in JX-1 cells than in JX-0 cells by measuring fluorescence intensity. After EGF pre-treatment, JX-1 and JX-0 cells were still sensitive to TG induced stimulation followed by the release Ca²⁺ with a similar pattern as to TG treatment alone, but a much higher level of intracellular Ca²⁺ intensity was found in JX-0 cells than in JX-1 cells. However, TG pre-treatment obviously depleted the intracellular Ca²⁺ storage in the endoplasmic reticulum of the cells resulting in the loss of responsiveness to EGF induction in both JX-1 and JX-0 cells. These results suggested that the antisense EGFR vector transfected JX-1 cells might possess an decreased intracellular Ca²⁺ pool content in endoplasmic reticulum of the cells under the control of an unknown cascade reactions originating from the interrupted EGFR expression and function triggered by antisense EGFR sequence. It has been shown that the sustained elevation of cytosolic Ca²⁺ is capable of inducing apoptosis[16]. On the other hand, alteration of intracellular Ca²⁺ pool content has been considered to be linked to the control of cell growth[17] as well as apoptosis[18, 19]. Our results in this report would support the notion that the alteration of intracellular Ca²⁺ pool content mediated by antisense EGFR sequence may interfere the signal pathways in regulating and enhancing the process of apoptosis in the JX-1 cells. Therefore, the link between EGF/EGFR signaling and intracellular Ca²⁺ level in the induction of apoptosis of JX-1 cells as well as the exact role played by antisense EGFR remain an attractive target for the future studies.

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REFERENCES

- [1] Williams GT, Smith CA. Molecular regulation of apoptosis: Genetic control on cell death. *Cell* 1993; **74**:777-9.
- [2] Barinaga M. Cell suicide: By ICE, not fire. *Science* 1994; **263**:754-6.
- [3] Ellis RE, Yuan J, Horvitz Hr. Mechanisms and functions of cell death. *Annu Rev Cell Biol* 1991; **7**:663-98.
- [4] Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; **267**:1456-62.
- [5] Grullick WJ. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. *Br Med Bull* 1991; **47**:87-98.
- [6] Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, et al. Close similarity of

- epidermal growth factor receptor and v-erbB oncogene protein sequences. *Nature* 1984; **307**:521-7.
- [7] Mendelsohn J. The epidermal growth factor receptor as a target for therapy with antireceptor monoclonal antibodies. *Semin. Cancer Biol* 1990; **1**:339-44.
- [8] Xu Y, Jiang W, Peng S. EGFR expression and EGF stimulation of proliferation in human liver carcinoma cells. *Acta Biol Exp Sin* 1989; **22**:445-53.
- [9] Xu Y, Jiang W, Peng S, Chen Y. Antisense EGFR sequence reverses the growth properties of human liver carcinoma cell line BEL-7404 *in vitro*. *Cell Res* 1993; **3**:75-83.
- [10] Rawson CL, Loo DT, duimstra JD, Hedsrom OR, Schmid ES, Barnes DW. Death of serum-free mouse embryo cells caused by epidermal growth factor deprivation. *J Cell Biol* 1991; **113**:671-80.
- [11] Wu X, Fan Z, Masui H, Rosen N, Medelsohn J. Apoptosis induced by an anti-epidermal growth receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest* 1995; **95**:1897-1905.
- [12] Fath I, Schweighoffer F, Rey I, Multon MC, Boiziau J, Duchesne M, Tocque B. Cloning of a Grb2 isoform with apoptotic properties. *Science* 1994; **264**:971-4.
- [13] Armstrong DK, Kaufmann SH, Ottaviano YL, Furuya Y, Buckley JA, Isaacs JT, Davidson NE. Epidermal growth factor-mediated apoptosis of MDA-MB-468 human breast cancer cells. *Cancer Res* 1994; **54**:5280-3.
- [14] Fu T, Xu Y, Jiang W, Zhang H, Zhu P, Wu J. EGF receptor-mediated intracellular calcium increase in human hepatoma BEL-7404 cells. *Cell Res* 1994; **4**:145-53.
- [15] Gu J, Liu H, Fu T, Xu Y. Thapsigargin increases apoptotic cell death in human hepatoma BEL-7404 cells. *Cell Res* 1995; **5**:59-65.
- [16] Lee S, Chris takos S, Small MB. Apoptosis and signal transduction: clues to molecular mechanism. *Current Opinion in Cell Biol* 1993; **5**:286-91.
- [17] Short AD, Bian J, Ghosh TK, Waldron RT, Rybak SL, Gill DL. Intracellular Ca²⁺ pool content is linked to control of cell growth. *Proc Natl Acad Sci USA* 1993; **90**:4986-90.
- [18] Lam M, Dubyak G, Chen L, Nunez G, Miesfeld R, Distelhorst CW. Evidence that Bcl-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca²⁺ fluxes. *Proc Natl Acad Sci USA* 1994; **91**:6569-73.
- [19] Kaneho Y, Tsukamoto A. Thapsigargin-induced persistent intracellular calcium pool depletion and apoptosis in human hepatoma cells. *Cancer Lett* 1994; **79**:147-55.

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