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Induction of Apoptosis by Selected Natural Products in v-Cbl Transformed COS and NIH 3T3 Cells

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Abstract: A comparative study was made on the relative sensitivity of normal cells vis-a-vis transformed cells to the induction of apoptosis by the selected natural products. Oncoproteins predispose cells for apoptosis. *Flourensia cernua, Ambrosia chamissonis* and *camptothecin*, was used as stimuli to induce apoptosis in transformed cells. V-Cbl plasmid -whole plasmid carring the v-Cbl gene-(pGEX-KG-v-Cbl 8,000 kb) and empty vector (pGEX-KG 5,000kb) was used for transfection of COS and NIH 3T3 cells. We hypothesized that the Cbl proteins could predispose the transformed cells to apoptosis, by making cells vulnerable to outside insult, could lead to their apoptotic death. Even with higher doses, such as 30 and 40 µL, of *Flourensia cernua*, apoptotic influence was not statistically significant -0.035% of all cells. Twenty microliter of *Ambrosi*a *chamissonis* also produced a statistically insignificant number -0.040% of all cells. The presence of intact nuclei stained pink by Propidium Iodide was generally observed, indicating necrosis. After longer duration (72 h), a higher number of necrotic cells was always observed. Murine fibroblast NIH 3T3 cells were more often found to be necrotic than COS fibroblasts. Delivering DNA by transfection resulted in toxicity, leading to the constant presence of necrosis.

Key wards: Apoptosis, fibroblast, natural products, necrosis, plasmid

INTRODUCTION

The strategy presented was a treatment using natural products to trigger apoptosis in normal and transformed COS and NIH 3T3 fibroblasts *in vitro*.

An alternative approach may come from the observation that oncoproteins overload neoplastic cells and deregulate the cells' life cycle by sensitizing them to the influence of outside agents. Therefore, oncogenic transformation can be thought of as a preapoptotic stage predisposing cells to apoptosis.

Perhaps this approach can provide an answer to one of the most important and curious medical questions: Why is cancer so rare when it is an omnipresent threat to all metazoans or, in other words, how is it counteracted with such remarkable efficacy? Even in long-lived, large organisms with some 10^{17} possible cellular targets, cancers arise on average less than once in every three lifetimes[1].

Oncoproteins exhibit interdependency. Each oncoprotein is influenced in some way by the properties of one or more others for its growth or death. A better understanding of oncoproteins and their interactions in apoptosis may well provide the basis for rational cancer therapies in the future.

The superfamily of growth factor receptor tyrosine kinases has received a great deal of research attention both because their importance for normal cell growth and their relevance to understanding the molecular events of cancer. The superfamily is further subdivided into a number of subfamilies based on structural motifs. The Epidermal Growth Factor (EGF) subfamily comprises the focus of research in our laboratory and includes the EGF receptor, the Neu tyrosine kinase (also referred to as ErbB2 or HER2), the ErbB3 (HER3) protein and ErbB4 (HER4) protein. These proteins are associated with breast cancers, lung, liver, stomach and other human malignancies such as adenocarcinomas and neuroblastomas.

Cbl was first identified as part of a transforming retrovirus which arose in a mouse pre-B cell lymphoma[2]. This retrovirus, named Cas-Br-M, was originally isolated from a wild mouse near Lake Casitas, California USA and induces T-and B-cell lymphomas with latent periods of 5-8 months[3]. During the study of this particular retrovirus Langdon added extract containing viral particles to

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mouse fibroblasts. Unexpectedly, the extract transformed the cells, indicating that the virus contains an oncogene which transforms murine fibroblasts[2] Langdon's laboratory named this oncogene v-Cbl for Casitas B-lineage lymphoma.

The c-Cbl proto-oncogene was identified as the cellular homologue of the v-Cbl oncogene isolated from mice that developed a pre-B cell lymphoma following infection with the replication-competent Cas-Br-M murine leukemic virus[2]. c-Cbl is expressed at relatively high levels in a wide range of hematopoietic tumor cell lines as well as in normal tissues such as thymus and testis[4]. The c-Cbl gene product has been identified as a 120 kDa nuclear protein with apparent DNA binding and dimerization domains characteristic of transcription factors[5]. A single c-Cbl locus termed Cbl2 has been mapped to human chromosome 11q23[6].

The c-Cbl protein is specifically phosphorylated by EGF, but not by heregulin, which stimulates the tyrosine kinase activity of Neu proteins.

v-Cbl is the carboxy-truncated form of c-Cbl and lacks the proline rich domain which is responsible for association with SH3 domains of signaling proteins. It also lacks the RING finger and the leucine zipper which are present in the cellular form. v-Cbl is localized to both the nucleus and the cytoplasm and binds DNA in a zinc-dependent manner, whereas c-Cbl is localized to the cytoplasm and has no transforming activity. However, c-Cbl becomes oncogenic after deletion of 17 amino acids c-terminal to the v-Cbl coding region. Therefore, it has been suggested that the c-terminal half of c-Cbl is responsible for its cytoplasmic retention and maintenance of its normal cellular function[7].

Although, the regulation of tyrosine phosphorylation of Cbl has been extensively studied in recent years, little is known about Cbl-b[8].

Two Cbl proteins, c-Cbl and Cbl-b, cytoplasmic form, each weighing approximately 120 kDa have been proven to be present in breast cancer and we also isolated them from five breast cancer cell lines: MDA-MB 231, MDA-MB 453, MDA-MB 468, BT 20 and SKBR 3[9].

In order to conduct experiments showing the influence of oncoproteins in apoptosis induction, we transformed COS and NIH 3T3 cells using v-Cbl plasmid (whole plasmid, 8,000 kbp), carring v-Cbl gene insert, (3,000 kbp) and pGEX-KG (empty vector, 5,000 kbp) (Fig. 1) . The constituents of DNA were the gifts of Dr. Richard Cerione (Cornell University, Ithaca N.Y.).

Selected phytochemical extracts (*Flourensia cernua* crude extract 1 mg/1 mL DMSO, *Ambrosia chamissonis* crude extract 1 mg/1 mL DMSO, *Camptothecin* 1 mg/1 mL DMSO and pure DMSO as control) were applied to

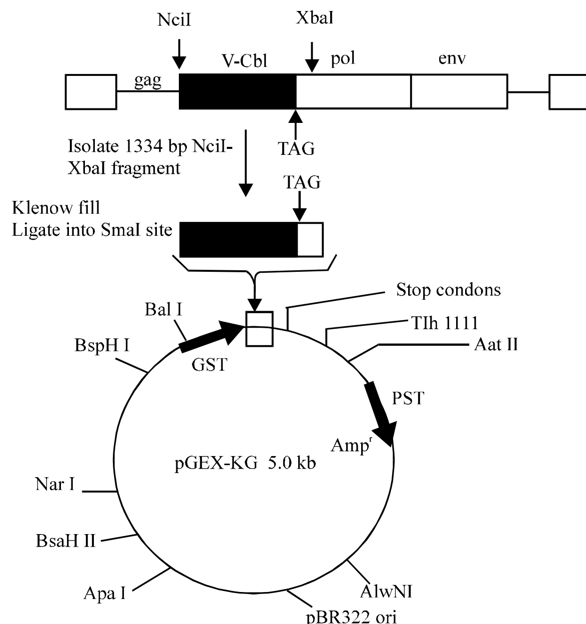

Fig. 1: Plasmid pGEX carrying the gene v-Cbl used for transfection of COS and NIH 3T3 fibroblasts

transformed and non-transformed cells *in vitro* to determine their apoptotic and necrotic effects[10]. These natural product were used as a stimuli to induce apoptosis.

A comparative study was made on the relative sensitivity of normal cells vis-à-vis transformed cells to the induction of apoptosis by the selective natural products.

MATERIALS AND METHODS

Cells: COS (African green mountain monkey kidney fibroblats) and NIH 3T3 (murine fibroblasts) from ATCC were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, D-5648) supplemented with 10% heat inactivated Fetal Bovine Serum (Atlanta Biologicals, Inc.) for COS cells or Calf Serum (Atlanta Biologicals, Inc.) for NIH 3T3 cells and Antibiotic/Antimycotic Solution 100x (Gibco BRL-1433). Cells were grown at 37°C, 5-7% CO_2 and 95% humidity. After reaching about 100% confluence, cells were trypsinized and centrifuged. During cell centrifugation (2,000 rpm for 2 min) cover slips (22 mm sq.) from Corning Glass Works were flamed. Cells were counted using a hemocytometer and delivered by resuspension to each well containing a cover slip at a concentration of 8,000 cells/well. Cells were allowed to adhere to cover slips for 24 h at 37°C, 5-7% CO_2. After 24 h transfection was completed and some plates with untreated cells were left as a control.

Plasmid purification: Transformed *Escherichia coli* expressing pGEX-KG-v-Cbl, a few colonies from a freshly streaked selective plate, were inoculated and grown in 500 mL LB plus Ampicillin (Sigma, A-9518) 100 mg mL^{-1} concentration. Incubation at 37°C. with vigorous shaking was conducted overnight. In the morning the bacterial cells were harvested by centrifugation at 6,000 rpm for 15 min at 4°C[11].

DNA Transfection: Six-well plates containing sterile cover slips in each well were prepared, some with 80% confluent COS cells and some with 80% confluent NIH 3T3 cells. After 24 h cells attached to the bottom and were ready for DNA transfection. Cells were transfected by using plasmids and Lipofectamine (Life Technologies, Inc.)

Immunoprecipitation and western blotting: After 48 h post-transfection c-Cbl expression was confirmed by Western blot analysis.

Each experiment was checked after 48 and 72 h and was repeated at least five times.

Extraction: Fresh leaves of *Flourensia cernua* were collected in November 2002 from Chihuahan Desert, Texas, USA. The collected material was stored in paper bag for easy absorption of moisture. After drying, leaves were ground to powder in Wiley Mill. Chloroform (1 L) and 100 g of dried plant material were mixed and left to solve overnight. On the next day, the product was partitioned into a methanol-insoluble portion. By drying methanol-insoluable fractions and crude extract in vacuum, pure extract was obtained for futher use with DMSO as a solvent. The same procedure was done with *Ambrosia chamissonis.* All concentrations were 10 mg/1 mL DMSO. As a commercial representative of natural products we used *Camptothecin* (Sigma C-9911), concentration 10 mg/1 mL DMSO.

RESULTS

The available data (12, 13) suggest and our present experiments confirm that untransformed fibroblasts COS and NIH 3T3, regardless of p53 status, are relatively resistant to apoptosis induction by selected natural products.

In a series of experiments which we conducted, plasmid pGEX-KG-v-Cbl (8,000 kb) encoding the viral gene v-Cbl and empty vector pGEX-KG (5,000 kb) were introduced to COS and NIH 3T3 fibroblasts .The ability of oncogenically transformed fibroblasts COS and NIH 3T3 to resist apoptosis triggered by selected natural products was the subject of our experiments.

For detection of apoptosis and necrosis *in vitro*, DNA Double Fluorescence Assay for Apoptosis Bloom was used (Personal comunication). The following stimuli were applied in this present experiments:

Stimulus *Flourensia cernua, Asteraceae*, crude extract, 10 mg/1 mL DMSO: Dosages of 10, 20, 30 and 40 μL were found to induce apoptosis without any sign of necrosis. Accordingly, we used the same dosages to attempt induction of apoptosis in COS and NIH 3T3 transfected fibroblasts .

Forty eight hour after transfection of COS and NIH 3T3 fibroblasts with 1 and 2 μL, respectively, of whole plasmid pGEX-KG-v-Cbl, apoptosis was not induced. With higher doses, such as 30 and 40 μL of *Flourensia cernua*, single apoptotic cells were present in the field, but were not statistically significant (0.035% of all cells). Each time, pinkish staining by Propidium Iodide was present, indicating necrosis. With increased dosages of *Flourensia cernua*, the number of necrotic COS and NIH 3T3 cells also increased. Untransfected COS and NIH 3T3 fibroblasts (regardless of p53 status) as a control did not show any sign of apoptosis induction by *Flourensia cernua* at any of the rates of application. The same results were achieved with pure DMSO. No signs of apoptotic induction were present.

The same procedures as above, when examined after 72 h showed no induction of apoptosis, but an increased number of necrotic cells (detached) was present.

After transfection with 1 or 2 μL, respectively, of empty vector pGEX-KG (5,000 kb) in COS and NIH 3T3 fibroblasts, apoptosis was not induced. Again, necrosis staining by Propidium Iodide was observed. Higher doses of *Flourensia cernua* proportionately produced a higher number of necrotic cells. The difference between the 48 and 72 h time periods shows a higher number of necrotic cells in longer duration of exposure to *Flourensia cernua*. Controls showed no sign of apoptosis or necrosis.

Stimulus: *Ambrosia chamissonis, Asteraceae*, crude extract 10 mg/1 mL DMSO: Dosages of 10 and 20 μL of *Ambrosia chamissonis* proved sufficient to induce apoptosis without necrosis in cancer cells. All experiments with *Ambrosia chamissonis* were conducted in the same manner as with *Flourensia cernua*: COS and NIH 3T3 cells were transfected with 1 and 2 μL, respectively, of pGEX-KG-v-Cbl plasmid and pGEX-KG empty vector for time periods of 48 and 72 h Controls were the same, with the stimulus being the only difference. All experiments provided results similar to those with

Flourensia cernua as stimulus. Accordingly, transformed fibroblasts (COS and NIH 3T3), after the influence of *Ambrosia chamissonis*, showed signs of necrosis when stained with Propidium Iodide. With the higher concentration of *Ambrosia chamissonis* (20 μL) a statistically insignificant number of apoptotic cells was sometimes detected (<0.040% of all cells). Necrosis was generally observed, as indicated by the presence of intact nuclei stained pink by Propidium Iodide.

Untransformed cells and cells treated with pure DMSO as control did not show any triggering of apoptotic changes. All cells were growing and attaching to cover slips. A statistically insignificant number of necrotic cells was found to be present in the control experiment. Observations after 72 h provided no results significantly different from those at 48 h. As might be expected, greater numbers of necrotic cells were observed after the longer duration. COS cells were more often attached to the cover slips and growing intact, whereas NIH 3T3 cells were more easily detached and necrotic in higher numbers.

DISCUSSION

Distinct cellular thresholds clearly exist for apoptosis induction[12,14]. Different cell types have different set points for triggering apoptosis. Certain oncoproteins like c-myc and viral E1A are effective inducers of apoptosis, particularly under certain conditions such as low serum concentration or high cell density[12].

Two different studies[15,16] proved suppression of apotosis by an oncogenic form of Cbl. Sinha *et al.*[15] reported that v-Cbl suppresses apoptosis and differentiation of 32Dcl3 cells trough enhancement of Lyn, Syk and PI3-kinase activities and of Bcl-2. Their observation that v-Cbl suppresses apoptosis of 32Dcl3 cells induced by growth factor withdrawal is corroborated by independent studies done by Anderson's laboratory. Both studies came to the same conclusion that Bcl-2 protein levels in oncogenic cells were overexpressed, but neither was able to determine a biological basis for this occurrence.

In the previous experiments, the untransformed fibroblasts COS and NIH 3T3 (regardless of p53 status) showed relative resistance to apoptosis induction by the natural products *Flourensia cernua* and *Ambrosia chamissonis*. In a series of experiments using COS and NIH 3T3 cells transformed by plasmid pGEX-KG-v-Cbl and vector pGEX-KG, we examined the ability of oncogenically altered cells to lower their apoptosis threshold to the influence of the natural products we selected.

Multiple factors are likely to modulate set points. One of present ideas was to test the sensitivity of transformed fibroblasts after transfection with v-Cbl, a viral gene producing oncoproteins. Since oncogenically transformed fibroblasts provide an experimental system analogous to naturally occurring tumors[13]. It was examined the effects of natural products on transformed fibroblasts expressing the Cbl oncoprotein. After transfection with whole plasmid v-Cbl and empty vector, induction of apoptosis in transformed COS and NIH 3T3 was not enhanced. Transfections with foreign DNA was toxic for cells, as indicated by the constant presence of pink staining by Propidium Iodide. Nuclei were stained, but always intact and without any morphological change, such as chromatin fragmentation, condensation, etc. As was expected, after 72 h more necrotic cells were detached and present in all cases than after 48 h. With increased dosage of extracts, the number of necrotic cells was also proportionately increased. Delivering DNA by transfection in any experiment resulted in toxicity, but not in the induction of apoptosis. In some way this makes sense, as some fragments of DNA can be built in the genome (for example, viral DNA), transforming cells but not leading to programmed cell death. DNA damage or acceptance of new fragments in the genome may still be critical for the induction of apoptosis, but would be unlikely to actually kill the cell.

Although the primary cellular targets of many anticancer agents have been identified, less is known about the process leading to the selective death of cancer cells[17] and almost nothing is known about the pathways which might be used by natural products or other agents in engendering apoptosis. A more complete understanding of cellular resistance to cancer therapy may require the elucidation of the mechanisms by which natural products or other agents can cause cell death. Since many chemotherapeutic agents induce DNA damage, the tumor-specific cytotoxicity of these agents has been attributed to their genotoxic effect on actively proliferating cells. However, in many cases, the cellular damage caused by active doses of these agents is not sufficient to explain their observed toxicity[18].

The relationship of tumor responsiveness to apoptosis underlies two important and humbling observations. Any success of previous cancer therapies may have been for different reasons than originally thought and the intrinsic killing power of the drugs or radiation employed may have been less important than their ability to trick the appropriate tumors into killing themselves. The notion that cancer therapy operates most potently through the induction of apoptosis should propel the study of programmed cell death and may imply

an enormous potential for the use therein of natural products.

The comparative study confirmed that untransformed fibroblasts COS and NIH 3T3 are relatively resistant to induction of apoptosis. Transformed fibroblasts produced apoptosis but to a statistically insignificant degree : 0.035% of all cells treated by *Flourensia cernua* and less than 0.040% of all cells treated by *Ambrosia chamissonis*.

Transformed fibroblasts showed sign of necrosis, with greater numbers of necrotic cells observed after longer durations. NIH 3T3 were more easily detached and necrotic to a greater degree than COS.

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