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CORRESPONDENCE

The anticancer plant stress–protein methyl jasmonate induces activation of stress-regulated c-Jun N-terminal kinase and p38 protein kinase in human lymphoid cells

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TO THE EDITOR

We have recently discovered that plant stress hormones, that regulate cell death in stressed plants, are capable of suppressing the proliferation and inducing apoptosis in various human transformed cells including leukemia, melanoma, prostate and breast cancer cells.[1] That study focused on several plant stress hormones, that is, salicylic acid and jasmonates. Methyl jasmonate (MJ) was determined to be the most potent agent. Furthermore, while MJ killed Molt-4 human lymphoblastic leukemia cells in an efficient manner, it did not affect normal human peripheral blood lymphocytes. Finally, MJ administered *in vivo* significantly increased the survival of lymphoma-bearing mice.[1]

c-Jun N-terminal kinase (JNK) and p38 are groups of mammalian mitogen-activated protein kinases (MAPK) that can be induced by cellular stress. JNK and p38 influence transcription activator protein 1 (AP-1) activity by both increasing the abundance of AP-1 forming proteins, and by stimulating their activity directly. AP-1 regulates the expression of various stress-related genes, including genes that mediate cellular injury (eg, IL-1β, IL-8, iNOS, etc). Stresses that induce JNK, p38 and AP-1 activities are numerous, for example, UV radiation, oxidative stress, heat shock, osmotic shock, cell stretching, pathogens, heavy metals and anticancer drugs such as taxol. The signaling pathways involving JNK, p38 and AP-1 mediate the death resulting from exposure to various stressors.[2,3]

Plant stress responses involve induction of cell death, and have recently been shown to upregulate MAPK and upstream enzymes, MAPK kinase kinase, in plant cells.[4,5] Therefore, we hypothesized that analogous to the induction of stress signaling in plant cells, the plant stress hormone MJ also induces stress signaling in human cells. To test this hypothesis, we analyzed stress-regulated MAPK signaling events in MJ-treated human Molt-4 leukemic cells and normal peripheral blood lymphocytes, and assessed the role of these signaling steps in MJ-induced cytotoxicity.

Molt-4 human lymphoblastic leukemia cells were exposed to the plant stress hormone MJ and the phosphorylation state of p38 and c-Jun was determined by Western blotting. c-Jun phosphorylation reflects the activity of the JNK MAPK, while p38 phosphorylation reflects its activation by upstream kinases. We administered MJ at 3 mM. This concentration was chosen based on our previous results showing that MJ at 3 mM induces strong cytotoxicity towards Molt-4 cells.[1] In addition, MJ is a plant stress hormone and we chose its concentration based on the plasma concentrations achieved upon administration of a well-studied plant stress hormone, salicylic acid. Salicylic acid is a nonsteroidal anti-inflammatory drug. While most nonsteroidal anti-inflammatory drugs (such as indomethacin) act in the micromolar range, salicylates act in the low millimolar range. The highest nontoxic pharmacological concentration of salicylate used in humans is approximately 3 mM. MJ induced time-dependent c-Jun phosphorylation that was inhibited by SB202190, a JNK inhibitor (Figure 1, upper panels). Similarly, MJ induced the phosphorylation of p38, again in a time-dependent manner (Figure 1, lower panels). Both signaling events were already detectable 10 min following exposure to MJ. The levels of nonphosphorylated c-Jun and p38 were practically unchanged throughout the experiment (Figure 1).

We further analyzed MJ-induced stress signaling by determining AP-1 activity in Molt-4 cells. To that end, we constructed a reporter plasmid expressing ECFP (a fluorescent protein) under the control of AP-1 (by inserting an AP-1 DNA-binding sequence: 5′-GAAATTCCAGAGAGTCATCAGAAGA-3′), and transfected Molt-4 cells with that plasmid. Fluorescence of these cells (Molt-4-AT) reflects AP-1-dependent ECFP gene transcription and translation. Molt-4-AT cells were treated with or without MJ or the classical AP-1 inducer TPA (as a positive control). Fluorescence microscopy revealed that while untreated cells did not exhibit fluorescence, both TPA- and MJ-treated cells were brightly fluorescent (Figure 2a). Clearly, this was an indication that MJ induced AP-1 activity in human Molt-4 leukemic cells. In addition, we evaluated induction of AP-1 activity upon exposure to MJ at different concentrations, for various time periods, using flow cytometry as shown in Figure 2b. The results show that MJ induced AP-1 activity in Molt-4-AT cells dose- and time-dependently. For example, only the highest concentration of MJ used (3 mM) induced some AP-1 activity within 2 h, while MJ at 0.5 mM was sufficient to induce AP-1 activity within 6 h. Thus, MJ induced JNK and p38 activities as well as the activity of AP-1, a transcription factor known to be regulated by these MAPK. In order to establish a cause-and-effect relationship between induction of MAPK and of AP-1 activities in MJ-treated cells, we employed inhibitors of JNK and p38. To that end, we determined AP-1 activity in MJ-treated Molt-4-AT cells in the presence or absence of SB203580 (a specific p38α and p38β inhibitor that does not inhibit the activity of JNK or of any other MAPK),[6] or SB202190 (an agent that inhibits JNK as well as p38α and p38β).[6] While SB202190 exhibited a strong inhibitory effect on AP-1 induction by MJ, SB203580 had no effect at all (Figure 2c). Moreover, while SB202190 was employed at 7.5 μM, we raised the concentration of SB203580 up to the maximal nontoxic level (25 μM) and still did not detect any inhibitory effect of the latter on the induction of AP-1 by MJ. Since a specific inhibitor of p38α and p38β (SB203580) was ineffective, whereas an inhibitor of p38α, p38β as well as JNK (SB202190) did suppress the induction of AP-1, we concluded that JNK mediates the induction of AP-1 activity by MJ.

MJ is selectively cytotoxic towards transformed cells while sparing normal lymphocytes.[1] Therefore, we decided to investigate whether the effect of MJ on p38 and JNK activities in normal lymphocytes differs from its effect on these enzymes in leukemic cells. To that end, we exposed peripheral blood lymphocytes from healthy individuals to MJ at 3 mM and measured the phosphorylation of c-Jun and p38. MJ

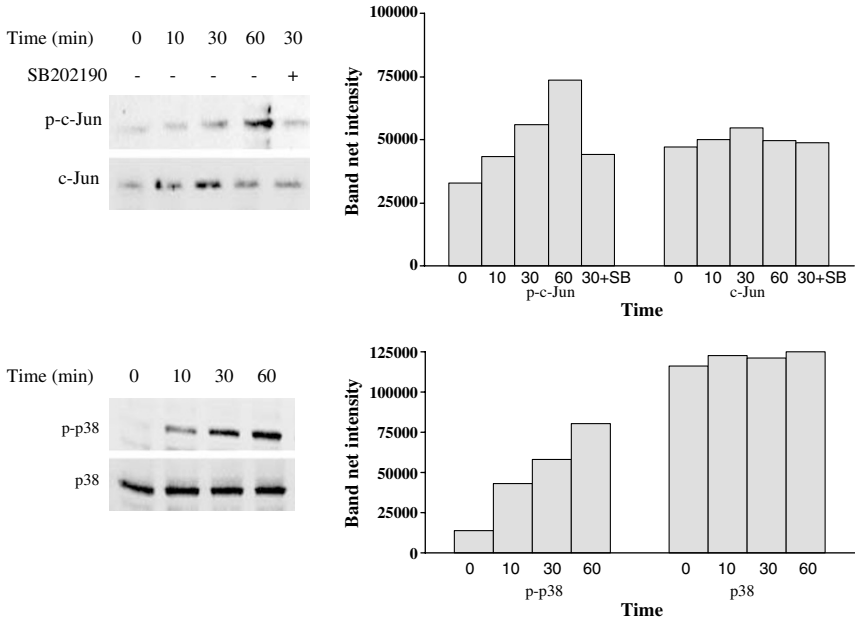

Figure 1 MJ induces c-Jun and p38 phosphorylation time-dependently in Molt-4 cells. Molt-4 cells (at 4×10^5/well) were seeded in six-well plates and treated with or without (0) MJ at 3 mM for the indicated periods of time (min). A sample was also prepared in which the JNK inhibitor SB202190 (SB), at 7.5 μM, was added 30 min prior to the addition of MJ. For analysis of phospho-c-Jun (p-c-Jun, reflecting JNK activity) and phospho-p38 (p-p38, indicating p38 activation) levels, whole-cell lysates were prepared and proteins were separated by SDS-PAGE, followed by immunoblotting using specific antibodies against p-c-Jun and p-p38. In addition, we performed immunoblotting using specific antibodies against nonphosphorylated c-Jun and p38 to control for the total levels of these proteins. Antigen–antibody complexes were stained with HRP-conjugated antibody and enhanced chemiluminescence reagent, and exposed to ECL film. Immunoblot images were digitized and the optical densities of specific antigen–antibody complexes were quantified. The intensity of bands generated with samples from untreated cells incubated for 60 min, was practically identical to that generated with samples at time 0. A typical experiment is shown, representing three experiments with similar results.

weakly induced c-Jun phosphorylation, to the same extent for 10–60 min approximately, and that was inhibited by SB202190 (Figure 3, upper panels). On the other hand, MJ strongly induced the phosphorylation of p38, with the highest response detected after 10 min and decaying thereafter (Figure 3, lower panels). The levels of nonphosphorylated c-Jun and p38 were practically unchanged. Thus, JNK activation appeared weaker in the normal lymphocytes (an increase of 23% in the normal cells *versus* an increase of 124% in the Molt-4 cells, after 60 min); and we detected the highest extent of p38 activation in the blood lymphocytes 10 min following treatment, whereas it increased for at least 60 min in the Molt-4 cells (Figure 1 *versus* Figure 3). We hypothesized that these differences may be the basis for the selective cytotoxicity of MJ towards the transformed Molt-4 cells. To test this hypothesis, we investigated the possible role of p38 and JNK in the cytotoxic effect of MJ towards Molt-4 cells. Molt-4 cells were exposed to MJ in the presence or absence of the p38α, p38β and JNK inhibitors, SB203580 and SB202190. We administered both inhibitors at the range of 5–25 μM. In all, 25 μM was the highest concentration that was not cytotoxic by itself. The inhibitors did not decrease the cytotoxic effect of MJ, even at the highest concentration employed. Our results suggest that while MJ induced p38 and JNK activities, these enzymatic activities do not mediate the cytotoxic effect of MJ. The inhibitors we used, SB203580 and SB202190, act selectively on the α and β, but not γ and δ, isoforms of p38.[6] Therefore, our conclusion that p38 does not mediate the cytotoxic effect of MJ should be restricted to the α and β isoforms of this enzyme. In general, p38, JNK and AP-1 signaling molecules may or may not mediate stress-induced cell death depending on various parameters, including the cell type, the stimulus and the state of the cell.[3] Furthermore, the results suggest that differences in the induction of JNK and p38 activities in transformed *versus* normal lymphocytes (Figures 1 and 3, respectively), do not offer a plausible explanation for the selective cytotoxic effect of MJ towards transformed lymphocytes.

MJ induces apoptosis in Molt-4 cells, and we found that the cytotoxic effect of MJ towards Molt-4 cells is independent of JNK and p38 activities. However, the induction of AP-1 by MJ in Molt-4 cells depends on JNK activity (Figure 2c). Thus, our results indicate that the transcription factor AP-1 does not mediate the apoptotic effect of MJ towards Molt-4 cells. This raises the possibility that the cytotoxic effect of MJ is independent of *de novo* RNA transcription altogether. Thus, we determined the extent of cytotoxicity induced by MJ in Molt-4 cells in the presence or absence of an RNA synthesis inhibitor, actinomycin D (Figure 4a), or a protein synthesis inhibitor, cycloheximide (Figure 4b). Since both inhibitors can by themselves induce apoptosis in Molt-4 cells, we administered actinomycin D and cycloheximide up to their highest non-apoptogenic concentrations. Neither inhibitor decreased the cytotoxic effect of MJ towards Molt-4 (Figure 4), indicating that the effect is independent of RNA and protein synthesis. To ensure that cycloheximide at the concentrations used by us could in fact prevent the *de novo* biosynthesis of cellular proteins putatively instrumental in the apoptotic process, we determined that cycloheximide effectively suppressed protein synthesis in the cells (Figure 4c).

We show that MJ induces JNK, p38 and AP-1 activities in human lymphoid cells, similar to the recently described ability of jasmonates to induce MAPK and MAPK kinase kinase

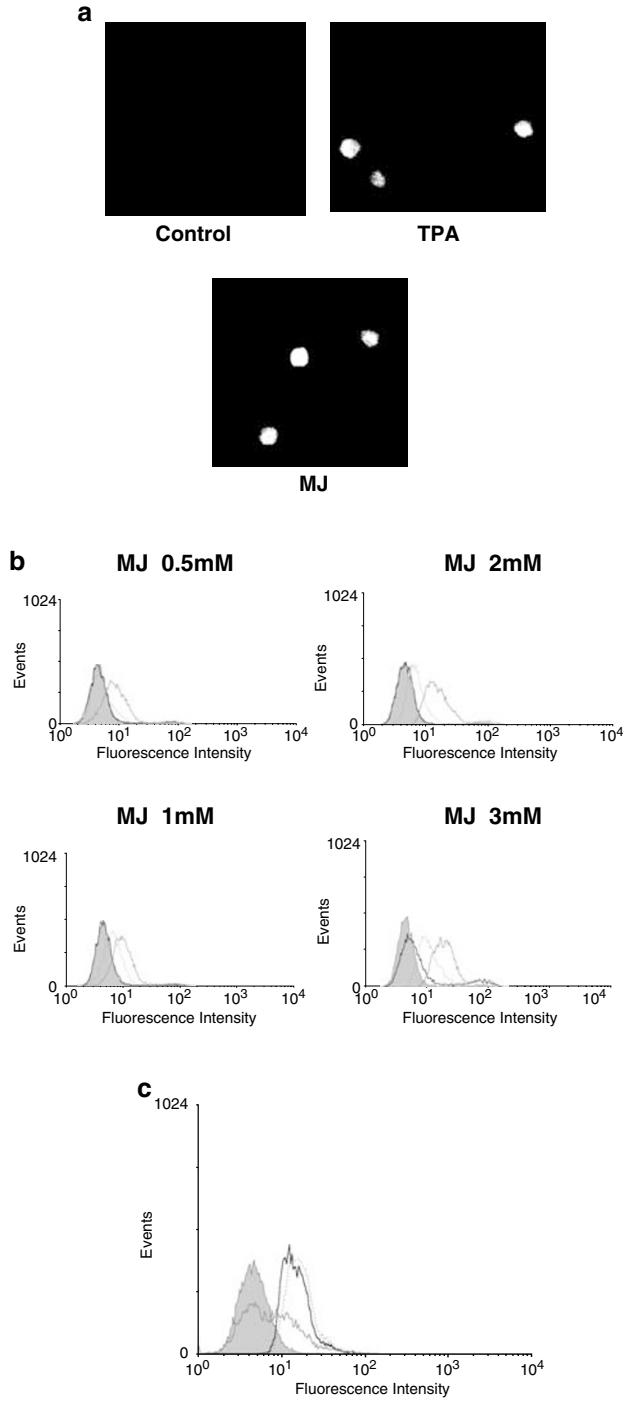

Figure 2 MJ induces AP-1 activity in Molt-4 cells. (a) Molt-4-AT cells (5×10^5) were treated with TPA (5 ng/ml) or MJ (3 mM), both for 6 h. Untreated cells served as a control. Cells were then washed, fixated and analyzed by fluorescence microscopy. (b) Molt-4-AT cells (5×10^5) were treated with MJ at the indicated concentrations. Cells were then washed, fixated and analyzed by flow cytometry. Dark gray area – untreated cells, black curve – cells treated for 2 h, discontinuous curve – cells treated for 4 h, gray curve – cells treated for 6 h. A curve situated further to the right along the *x*-axis represents cells exhibiting stronger fluorescence, that is, higher level of AP-1 activity. (c) Molt-4-AT cells (5×10^5) were treated for 6 h with MJ with or without SB203580 or SB202190. The inhibitors were added 30 min prior to the addition of MJ. Cells were then washed, fixated and analyzed by flow cytometry. Dark gray area – untreated cells, black curve – cells treated with MJ at 3 mM, discontinuous curve – cells treated with MJ at 3 mM + SB203580 at 25 μM, gray curve – cells treated with MJ at 3 mM + SB202190 at 7.5 μM.

activities in plants.[4,5] Thus, jasmonate plant stress hormones are capable of inducing analogous stress signaling pathways in evolutionarily remote cells, suggesting that stress-regulated MAPK signaling has been conserved over extremely long evolutionary distances. This reflects probably the fundamental role that stress responses play in the ability of organisms to survive environmental challenges.

Activation of the JNK and p38 MAPK pathways results in induction and enhancement of AP-1 activity.[2,3] Our findings indicate that while both JNK and p38 are activated by MJ, only JNK mediates the MJ-induced AP-1 activity. Four isoforms of p38 (α, β, γ and δ) have been identified,[3] and our antiphospho-p38 antibodies can interact with each of these isoforms. Recently, AP-1 activity in breast cancer cells was found to be elevated by the p38 α and β isoforms, but not by the γ or δ ones.[7] On the other hand, green tea polyphenol increases AP-1 activity in keratinocytes via p38δ.[8] Thus, different p38 isoforms may have different effects on AP-1 activity, and this may depend on the type of cell involved. Therefore, the fact that MJ induces p38 activation in Molt-4 cells, but this signaling event does not mediate enhancement of AP-1 activity, may reflect a selective activation of specific p38 isoforms that in these cells do not lead to enhanced AP-1 activity. Notably, the specific p38 inhibitor we used, SB203580, acts selectively on the α and β, but not γ and δ, isoforms of p38.[6] It therefore follows that one of the following interpretations may explain the lack of effect of SB203580 on MJ-induced AP-1 activity in Molt-4 cells: (a) MJ induces p38α and/or p38β and these isoforms do not induce AP-1 activity in this type of cells, that is, p38 does not induce AP-1 activity in Molt-4 cells; or (b) MJ induces p38γ and/or p38δ, in which case our results do not resolve the issue of whether p38 induces AP-1 activity in Molt-4 cells. It will be possible to address this question once inhibitors of p38 γ and δ become available.

Since we found that while JNK mediates the induction of AP-1 activity by MJ, it does not mediate the cytotoxic effect of MJ on Molt-4 cells, our results support the suggestion that the JNK-AP-1 signaling axis is not essential for the cytotoxic effect of MJ. As for the mechanism mediating MJ cytotoxicity, we obtained recently evidence suggesting that mitochondria are involved in the mechanism of action of MJ cytotoxicity, that is, MJ induces perturbation of mitochondrial function in cancer cell lines (Flescher E, personal communication, 2003). Moreover, we performed preliminary studies on primary leukemia cells from CLL patients and found that jasmonates induce death and mitochondrial membrane depolarization in these cells (Flescher E, personal communication, 2003).

MJ-induced cytotoxicity appears to be AP-1-, RNA and protein synthesis independent. It is possible that AP-1 activity is essential in instances where apoptosis is *de novo* protein synthesis dependent, in order to turn on expression of genes that are required for the apoptotic process. On the other hand, AP-1 activation may not be required in apoptotic death scenarios that are *de novo* protein synthesis independent, although AP-1 may be activated in these cases as well. Thus, the death process in MJ-treated Molt-4 cells appears to be activated in the absence of new gene expression.

In conclusion, the plant stress hormone (and potent anti-cancer agent) MJ can induce death in human leukemic cells as well as activate two stress-regulated MAPK: JNK and p38. MJ-induced JNK activity results in AP-1 activity. However, the cytotoxic effect of MJ is MAPK, RNA synthesis and protein synthesis independent. It thus follows that MJ induces two independent processes in Molt-4 leukemic cells: death and a typical stress response. MJ kills selectively transformed cells,[1] and we report here that the stress signaling induced by MJ in

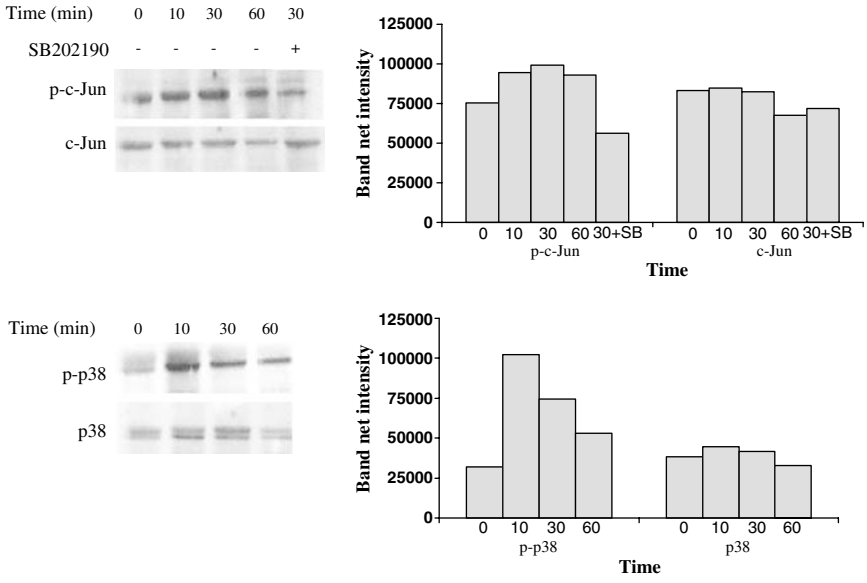

Figure 3 MJ induces short-lived p38 phosphorylation in peripheral blood lymphocytes. Peripheral blood lymphocytes (at 4×10^5/well) were seeded in six-well plates and treated with or without (0) MJ at 3 mM for the indicated periods of time (minutes). A sample was also prepared in which the JNK inhibitor SB202190 (SB), at 7.5 μM, was added 30 min prior to the addition of MJ. For analysis of phospho-c-Jun (p-c-Jun, reflecting JNK activity) and phospho-p38 (p-p38, indicating p38 activation) levels, whole cell lysates were prepared and proteins were separated by SDS-PAGE, followed by immunoblotting using specific antibodies against p-c-Jun and p-p38. In addition, we performed immunoblotting using specific antibodies against nonphosphorylated c-Jun and p38 to control for the total levels of these proteins. Antigen–antibody complexes were stained with HRP-conjugated antibody and enhanced chemiluminescence reagent, and exposed to ECL film. Immunoblot images were digitized and the optical densities of specific antigen–antibody complexes were quantified. The intensity of bands generated with samples from untreated cells incubated for 60 min, was practically identical to that generated with samples at time 0. A typical experiment is shown, representing three experiments with similar results.

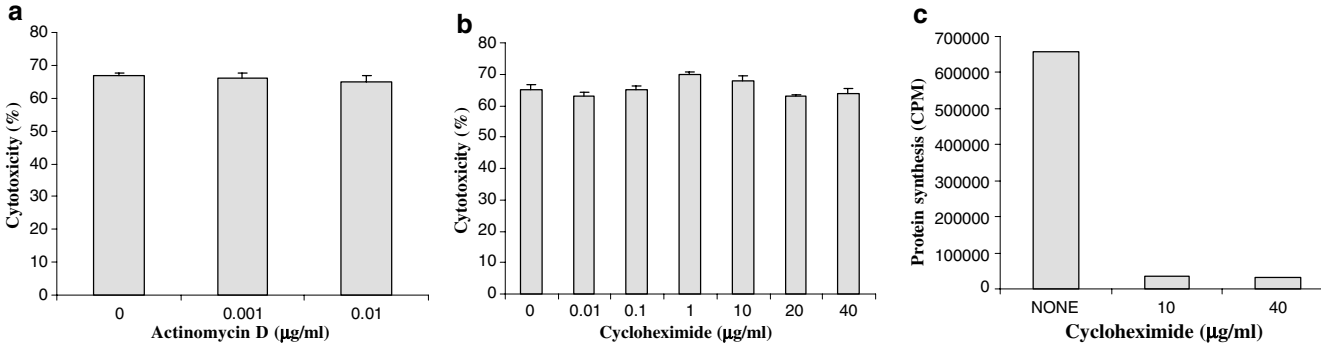

Figure 4 RNA and protein synthesis inhibitors do not suppress the cytotoxic effect of MJ towards Molt-4 cells. Molt-4 cells (at 1.5×10^4/well) were seeded in 96-well plates. MJ at 3 mM was added for 24 h with or without actinomycin D (a) or cycloheximide (b), at the indicated concentrations. The inhibitors were added 30 min prior to the addition of MJ. Optical density representing viable cells was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. Results are presented as means $\pm$ s.d., $n = 3$. Also, protein synthesis was determined by [^{35}S]methionine incorporation in Molt-4 cells incubated with or without (None) cycloheximide at the indicated concentrations (c).

normal lymphocytes differs (by extent and kinetics) from that induced in leukemic cells. Therefore, we hypothesize that while stress-regulated MAPK signaling does not mediate MJ-induced leukemic cell death, the different characteristics of these signaling events in transformed *versus* nontransformed lymphocytes, reflect a different manner through which these respective cell populations perceive MJ-induced stress in general. Thus, this different perception is not restricted to MAPK signaling, and may contribute to the fact that only transformed cells are susceptible to the cytotoxic effects of jasmonates. Normal lymphocytes, on the other hand, can survive the stress due, possibly, to a more flexible capability to overcome environmental challenges. Numerous plant-based chemicals exhibit anticancer activities and some of these compounds inhibit signal transduction in cancer cells. To the best of our knowledge, we present here for the first time evidence that a plant-derived anticancer agent induces similar signaling in mammalian cancer cells, and in its natural target – plant cells.[4,5] Therefore, knowledge about the ability of plant molecules to modulate signal transduction in plant cells, may be the basis for a novel approach towards identifying compounds capable of modulating signaling in human cancer cells.

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R Rotem[1]
O Fingrut[1]
J Moskovitz[2]
E Flescher[1]

[1]*Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel;*
[2]*Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA*

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Gene expression profile unravels significant differences between childhood and adult Ph+ acute lymphoblastic leukemia

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TO THE EDITOR

The t(9;22) translocation is identified in about 3–5% of children with acute lymphoblastic leukemia (ALL)[1,2] and in about 25% of adult patients.[3] In both children and adults with ALL, the t(9;22) presence is associated with a high risk for treatment failure.[4,5] However, recent retrospective studies showed that Ph+ childhood ALL is a heterogeneous disease with regard to treatment response.[1,2,5] High leukocyte count, old age and poor response to the prephase treatment with prednisone and intrathecal metotrexate influence treatment outcome.[1,2,5] Patients with prednisone good response (PGR) had a significantly lower risk of treatment failure compared to those with prednisone poor response (PPR), when treated with intensive Berlin–Frankfurt–Münster (BFM) chemotherapy, whether associated with bone marrow transplantation (BMT) or not.[1] PPR children present an equally poor clinical response and prognosis as Ph+ ALL adults.[6] Recent studies on gene expression profile in childhood and adult ALL[7,8] distinguished gene signatures specific to genetic subclasses. However, Ph+ ALL showed the weakest signature among genetically distinct subclasses, indicating high heterogeneity, which correlates with the clinical variability.

In an attempt to dissect the heterogeneity in Ph+ acute leukemia, we studied the expression levels at diagnosis of a selected number of genes that had emerged as being particularly significant from the two Ph+ gene expression profile studies published so far.[7,8] We limited the analysis to genes involved in cell cycle, apoptosis or stress response. Among the 10 genes whose expression was specific enough to discriminate childhood Ph+ leukemia from other genetic subclasses,[7] we selected four related genes: mitogen-activated protein-kinase-activated protein kinase 3 (MAPKAPK3), Cyclin D2 (CCND2), caspase 8 (CASP8) and caspase 10 (CASP10). A further five genes: histone-deacetylase 2 (HDAC2), minichromosome maintenance, S. Pombe, homolog of 6 (MCM6), microtubule affinity-regulating kinase 3 (MARK3), beclin 1 (BECN1) and telomerase protein component (TEP1), were selected from those that presented the highest different ratio of expression in adult Ph+ ALL resistant or sensitive to the tyrosine-kinase inhibitor STI571.[8] Using this approach, we analyzed bone marrow (BM) samples from 26 children (under 14 years of age) and nine adults with Ph+ ALL; BM- and PB-mononuclear cells (MNC) from eight and five healthy volunteers, respectively, were also studied.

The diagnosis of ALL Ph+ was based on morphological, cytochemical, immunophenotypic and molecular analysis. Children were treated according to the different consecutive protocols of the Associazione Italiana Ematologia Oncologia Pediatrica (AEIOP), whereas adult patients received protocols of the Northern Italy Leukemia Group (NILG). It is worth noting that two pediatric patients were identical twins (manuscript in preparation), and showed a different response to prednisone therapy. According to the BFM criteria, PGR was defined as the presence of less than 1000 lymphoblasts/μl blood after the first 7 days of prednisone therapy, and one intrathecal injection of age-adapted dose of MTX on day 1. Those with more than 1000 lymphoblasts/μl blood on day 8 were classified as prednisone poor responders (PPR).

BM and PB mononuclear cell preparations were obtained by Ficoll–Hypaque centrifugation, and contained more than 98% of leukemia blasts, as assessed by morphological evaluation. RNA was extracted from cryopreserved samples and from the cell line TOM1, by ultracentrifugation using CsCl gradient method. The cDNA reverse transcription was performed using 1,0 μg RNA and SuperscriptII enzyme (Invitrogen, Carlsbad, CA, USA) in 20 μl final volume. The resulting cDNA was subjected to qualitative or quantitative PCR. For the diagnosis of the t(9;22) translocation, we used qualitative