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DIFFERENTIATION-INDUCING POTENCY OF THE SECO-STEROID JK-1624F2-2 CAN BE INCREASED BY COMBINATION WITH AN ANTIOXIDANT AND A p38 MAPK INHIBITOR WHICH UPREGULATES THE JNK PATHWAY

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Abstract

Low calcemic analogs of vitamin D are candidates for differentiation therapy of human myeloid leukemias. We report here that the seco-steroid synthesized to have resistance to intracellular degradation and low calcemia-inducing activity, 1 α -hydroxymethyl-3 β -16-ene-24,24-difluoro-25-hydroxy-vitamin D₃ (JKF), induces monocytic differentiation in four established human myeloid leukemia cell lines, HL60, U937, THP-1, NB4, and murine myeloid leukemia cells WEHI-3B D⁺. JKF has differentiation-inducing potency which is slightly lower than the physiologically active form of vitamin D, 1,25(OH)₂vitamin D₃ (1,25D). However, simultaneous addition of carnosic acid (CA), an antioxidant, and SB20190 (SB), an inhibitor of p38 MAP kinase, increases the differentiation efficiency of JKF to a level similar to the level observed when 1,25D is used in such combinations. We also show for the first time that SB inhibits the phosphorylation of MAPKAPK-2, a downstream target of p38MAPK, but upregulates the phosphorylation of at least one of the isoforms of JNK (p46 JNK1) and of c-jun in all four human myeloid cell lines studied here. These studies indicate that the JNK1 pathway is positively associated with monocytic differentiation of several subtypes of myeloid leukemia cells arrested at different developmental stages. Further, since JKF is less calcemic than 1,25D, the data suggest that JKF combined with CA and SB is likely to have a therapeutic advantage over 1,25D-based experimental regimens for myeloid leukemias.

Keywords

Vitamin D; Myeloid Leukemias; Differentiation; MAPK; p38; JNK; c-jun; ATF-2

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1. Introduction

Major advances in the treatment of some subtypes of human leukemias have recently been made [1-4], yet these diseases continue to have grim prognosis, and additional novel approaches to therapy are still needed. Since a large proportion of myeloid leukemias are characterized by a block to differentiation into the mature, functional hematopoietic cells, the induction of differentiation seems to be a logical component of such therapy. Indeed, treatment with all-trans retinoic acid (ATRA) is remarkably effective in one subtype of myeloid leukemia, the acute promyelocytic leukemia (APL, or M3 AML) [1,2,5,6], and the physiological form of vitamin D, 1 α ,25-dihydroxyvitamin D₃ (1,25D), is an effective differentiation agent in model systems [7-10].

Although 1,25D is also being explored as a therapeutic agent in malignancies other than leukemias [11-13], the principal problem in translating the findings with 1,25D to the clinic is that doses effective for induction of differentiation disturb calcium homeostasis in vivo, leading to life-threatening hypercalcemia. One approach to overcome this problem is to design analogs of 1,25D with high potency as differentiation agents, but with reduced calcemia-inducing properties. For instance, the introduction of the D ring 16 unsaturation, and fluorines into the side chain of the seco-steroids, decreases their susceptibility to initiation of degradation by the CYP24 hydroxylase [14-17], while the introduction of the 1 α -hydroxymethyl group in the A ring serves to reduce the calcemic properties of the compound [18]. One such synthetic derivative of 1,25D, compound JK-1624F2-2 (JKF), shown in Fig. 1, has been safely used in animal studies [19], but its effect as a differentiation-inducing agent has not been previously evaluated.

This laboratory has been advocating an additional approach to the realization of the potential of 1,25D as an antileukemia therapeutic agent. In a series of studies we reported that the antileukemic effects of 1,25D are enhanced by combining 1,25D with carnosic acid (CA), a plant-derived antioxidant polyphenol [20,21], and that the potency of the combination can be further enhanced by adding SB202190 (SB), an anti-inflammatory drug that acts by inhibiting the kinase activity of the stress-related MAP kinase, p38 [22-24]. We now report that JKF has differentiation-inducing activity on five myeloid leukemia cell lines studied here, and its potency can be increased by combination with CA and SB. Further, while the stress-related pathways p38MAPK and JNK are upregulated during 1,25D- and JKF-induced differentiation to a similar if not identical extent, the levels of activated ATF-2, a downstream target of these pathways, are reduced by the JKF-containing differentiation “cocktail”.

2. Materials and Methods

Cell culture and treatments

The cell lines, HL60-G [25], a subclone of human promyeloblastic leukemia HL60 cells, human myelomonocytic leukemia U937, acute monocytic leukemia THP-1 and acute promyelocytic leukemia NB-4 were maintained in RPMI 1640 medium (Mediatech, Washington, DC), supplemented with 1% glutamine and 10% heat-inactivated, iron-enriched bovine calf serum (HyClone, Logan, UT) in a 37°C incubator supplied with 5% CO₂. Murine model of myelomonocytic leukemia, WEHI-3B D⁺ cells, were grown in McCoy's 5a medium plus 10% heat-inactivated fetal calf serum (GIBCO-BRL, Gaithersburg, MD) [28]. The cells were passaged and fed with medium 2-3 times per week to maintain the log phase growth. 1,25D was a kind gift from Dr. Milan Uskokovic, BioXcell, Inc., Nutley, NJ., and its analog JKF was synthesized as previously described [17,26]. Carnosic acid (CA) was purchased from Alexis Corporation (San Diego, CA.) The p38 MAPK inhibitor SB 202190, obtained from Sigma (St. Louis, MO), was added to the cultures 1 h before the exposure to CA, and CA was added to the cultures 1 h before the exposure to 1,25D. Cell viability was determined using trypan blue

(0.25%) exclusion, and cell numbers by counting cells in a Neubauer hemocytometer. Cell viability was also obtained by using gating in an Epics XL-MCL flow cytometry instrument (Beckman Coulter, Fullerton, CA), based on forward and side scatter [27]. Each experiment was repeated at least three times.

Determination of differentiation and cell death markers

Aliquots of 10^6 human leukemia cells were harvested at the indicated times, washed twice with $1 \times$ PBS, then incubated for 45 min at room temperature with 0.5 μ l MY4-RD-1 and 0.5 μ l MO1-FITC antibodies (Beckman Coulter), to detect the expression of surface cell markers CD14 and CD11b, respectively. The cells were then washed three times with ice cold $1 \times$ PBS, and resuspended in 0.5 ml $1 \times$ PBS. Two-parameter analysis was performed using the same XL-MCL flow cytometer described above. Isotypic mouse IgG1 was used to set threshold parameters. In addition, monocytic differentiation was monitored by cytochemical determination of nonspecific esterase (NSE) activity, as described previously [29]. The percentage of NSE positive cells in a total of 500 cells was counted using a hemocytometer. Differentiation of WEHI-3B D⁺ cells was determined by the nitroblue tetrazolium (NBT) reduction test, as previously described [28]. To determine the mode of cell death, annexin V and propidium iodide (PI) staining assay was performed (Annexin V-FITC Apoptosis Detection Kit, Sigma). Briefly, 0.5×10^6 cells of each group were suspended in 500 μ l $1 \times$ binding buffer, 5 μ l of annexin V-FITC and 10 μ l of propidium iodide were added to each cell suspension and incubated at room temperature for exactly 10 min. The fluorescence of the cells was determined immediately with the flow cytometer. Each experiment was repeated at least three times.

Cell extracts and Western blotting

Approximately 10^7 cells were spun down and washed 3 times with $1 \times$ phosphate buffered saline (PBS). The supernatants were then aspirated, and 100 μ l $1 \times$ lysis buffer (Cell Signaling, Danvers, MA) added to each pellet. Whole cell proteins were extracted and immunoblotting was carried out according to the manufacturer's protocol (Cell Signaling). Briefly, 40 μ g protein extract from cells were boiled in $3 \times$ sample buffer (125 mM Tris-HCl, pH 6.8, 1% SDS, 2% β -mercaptoethanol, and 0.01% bromophenol blue) for 5 min and loaded onto a 10% SDS-polyacrylamide gel. The separated proteins then were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Anti-P-p38 (Thr 180/Tyr 182), anti-P-MAPKAPK2 (Thr 334), anti-P-ATF-2 (Thr 71), anti-P-JNK (Thr 183/Tyr 185), and anti-P-c-jun (Ser 63) antibodies were purchased from Cell Signaling. Antibodies detecting total p38, JNK, MAPKAPK2, Crk-L were purchased from Santa Cruz Inc. (Santa Cruz, CA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% (w/v) Tween 20 for 1 h at room temperature, probed for 1 h with the specified primary antibodies mentioned above, and incubated with a horseradish-linked secondary antibody for 1 h. Bands of proteins on the membrane were visualized using the chemiluminescence assay system from Amersham. The membranes were then stripped according to the manufacturer's protocol (Amersham), and reprobed with a control antibody, usually Crk-L. The optical density (O.D.) of each band was quantitated using an image quantitator (Molecular Dynamics, Sunnyvale, CA). All computations were performed with an IBM-compatible personal computer using Microsoft Excel.

3. Results

3.1. Carnosic acid and SB202190 increase the expression of cell surface differentiation markers induced by 1,25D and its analog JKF

Preliminary to further translational studies of the effects of vitamin D derivatives (deltanoids) on freshly obtained blood samples from patients with myeloid leukemias, we compared the expression of various markers of differentiation in four human myeloid leukemia cell lines, representing cells arrested at different developmental stages of hematopoiesis. We first focused on CD14 and CD11b, cell surface proteins that function in mature monocytes and macrophages; CD14 serves as a receptor for the LPS-associated protein [30], while CD11b is a part of the integrin complex that functions in adhesion to other cells or surfaces [31]. Fig 2 shows that the upregulation of these markers by JKF was slightly less marked than by the equimolar concentration (10 nM) of 1,25D in all four cell lines. Although there was some variability in the extent to which the differentiation potency of JKF was lower (e.g. minimal in HL60 and THP-1 cells and quite marked in U937 cells), cumulatively the data in Fig 2 demonstrate that at submaximal concentrations JKF is less potent than 1,25D.

We have previously reported that 1,25D-induced differentiation of HL60 cells can be potentiated by the addition of antioxidants such as carnosic acid (CA), and an inhibitor of p38MAPK, SB202190 (SB) [22-24]. Since it seems important to establish the generality of these findings in myeloid leukemias, we compared the effects of these compounds on 1,25D and JKF-induced differentiation of the four cell lines (Fig 2).

Alone, CA had no significant effect on these surface markers of differentiation, and while SB induced a very modest upregulation of CD11b only in THP-1 cells, together with CA it appeared to upregulate the expression also of CD14 in most cells lines, though the extent was not sufficient to reach statistical significance. Importantly, both CA and SB increased the expression of these surface markers induced by 1,25D and by JKF in all cells lines, though SB was more effective in this respect than CA in all cell lines except HL60. Similarly, both CA and SB potentiated the differentiating actions of 1,25D and JKF in WEHI-3B D⁺ cells, a murine model of myeloid leukemia (data not shown). Added together, CA and SB, increased the deltanoid-induced expression of CD11b and CD 14 even more, suggesting that all these compounds are acting by different though complementary mechanisms. Of note, JKF supplemented with CA and SB induced CD11b and CD14 to an extent similar to that seen in cells treated with 1,25D in triple combination (last columns in all panels in Fig 2). Thus, when supplemented by CA and SB, JKF may be a differentiating agent for leukemia cells approximately equipotent with 1,25D in human and murine myeloid leukemia cells, yet reportedly less calcemic [19,32].

3.2 Effects of carnosic acid and SB202190 on the intracellular differentiation marker “nonspecific esterase”

Since there is no available comparative study of the concordance of different markers of myelomonocytic differentiation, we also determined in this system the induction of an intracellular enzymatic reaction associated with the mature phenotype of cells that phagocytose extracellular material. Such an enzyme, frequently used as a marker of monocytic differentiation in human hematopoietic cells, is the so-called “non-specific esterase” (NSE), presumably helpful for the digestion of phagocytosed intracellular material. Since this reaction, enumerated in individual cells by microscopic examination, accumulates fewer events than flow cytometry, it tends to be less accurate than the determination of CD11b/CD14. Nonetheless, Fig 3 shows that the conclusions reached by the examination of the surface markers CD11b /CD14 are in general confirmed by NSE positivity, given the lower accuracy of this method.

It can also be noted that the NSE enzyme is present in a high proportion of U937 and THP-1 cells in basal state, i.e. not exposed to differentiating agents, but degrees of reaction intensity can be clearly distinguished by microscopic examination, and enumeration of strongly reacting cells (NSE⁺⁺) can supplement the assessment of differentiation in these cells (Fig 3, panels B and C). Intriguingly, the proportion of NSE positive cells increased when exposed for 72 hours to CA alone, especially in NB4 cells (Fig 3, panel D). This reaction also increased in 1,25D or JKF-treated cells, alone or cotreated with CA, but SB decreased the effect of such treatments. This was apparent to a varying extent in all four human cell lines studied here (Fig 3). Similarly, in WEHI-3B D⁻ cells the NBT reaction induced by deltanoid/CA combinations was reduced by SB (not shown).

3.3. Reduced cell viability following supplementation of deltanoid treatment with CA and/or SB

Exposure to 1,25D, JKF, CA or SB added individually had only very minor or no effect on the viability of the cells studied here. However, the “triple combination” of a deltanoid, CA and SB (DCS), which was found to be the most potent for an induction of surface markers of differentiation (Fig 2), also appreciably reduced the viability of all cell lines studied here as seen by estimation of cell viability by light scatter gating using a flow cytometer (Fig 4), and similar results were observed by trypan blue exclusion (data not shown). This effect of DCS varied in intensity in different cell lines, being minimal in HL60 cells, and more marked in U937, NB4 cells (Fig. 4), as well as WEHI-3B D⁻ cells (data not shown). Cell death appeared to be due predominantly to necrosis, though an apoptotic subpopulation of dead cells was also detected (Fig 5). Since the number of cells positive for the NSE reaction was also relatively reduced in the “triple combination” treatment groups (Fig 3), this raises the possibility that the cell death signals enhanced by the “triple combination” are inhibitory to the pathways that signal deltanoid-induced upregulation of the NSE reaction. Thus, induction of cell death may be another therapeutic option when this combination of agents is used, and appears to be cell type and perhaps pathway specific.

3.4. The p38MAPK inhibitor SB202190 can increase phosphorylation but reduces the level of p38MAPK protein

It has been observed that the inhibition of p38MAPK enzymatic activity by SB can paradoxically increase its phosphorylation [23,24,33], and this was confirmed here (Fig 6, top panels in each cell line grouping). Interestingly, however, the steady-state level of p38MAPK protein was reduced in all cell lines examined (Fig 6, panels second from the top). As expected, the hyper-phosphorylated form of MAPKAPK2 (K2), the upper band (or its portion, if incompletely resolved on the gel) in the third panel in each grouping, was not present in SB-treated cells, consistent with effective inhibition of p38MAPK activity, and with K2 being a direct target of p38MAPK. The loss of this phosphorylated component of the K2 band was apparent irrespective of whether SB was used alone or in combination, and was most obvious in THP-1 cells. Collectively, these data suggest that the inhibition by SB of p38MAPK enzymatic activity leads to (1) its compensatory phosphorylation, probably due to the loss of negative feedback of an upstream kinase [23], and (2) reduced stability of the p38MAPK protein. The markedly reduced levels of total p38MAPK protein in SB-treated cells may contribute to the reduced activation of the down-stream targets of p38MAPK, such as K2.

3.5. Activating phosphorylation of c-jun correlates with the expression of surface differentiation markers

When the activating phosphorylations of JNK pathway, the other arm of stress-related signaling [34] were examined, the striking observation was the marked activation of at least one isoform of JNK regulatory protein [35,36] in cells treated with SB, alone or in collaboration with other

compounds (Fig 7). Interestingly, while the increased activating phosphorylation was seen in all cell lines examined here, the extent of phosphorylation of the isoforms varied in the different lines. For instance, in HL60 cells both p46 JNK (mainly JNK1 isoforms) and p54 JNK (mainly JNK2 isoforms) were intensely phosphorylated only in cells exposed to SB, while in THP-1 cells p54 JNK was highly phosphorylated already in the basal state, and only the hyperphosphorylated form of p46 JNK showed a marked increase following exposure to SB (Fig 7). The hyperphosphorylated p46 JNK was also increased in U937 and NB4 cells exposed to SB, and correlated best with the presence of the activating phosphorylation of c-jun. In turn, the levels of P-c-jun correlated well with optimal expression of surface markers of differentiation (CD11b/CD14) in all cell lines (compare Figs 2 and 7). It is clear, however, that deltanoids also activate pathways other than JNK/c-jun that are required for the expression of surface markers of differentiation, since addition of 1,25D or JKF to the CA/SB combination markedly increased CD11b/CD14 expression, but hyperphosphorylation of p46 JNK was similar in intensity in all SB-treated cells (Fig 7). Curiously, although the intensity of JNK phosphorylation in cells treated with SB alone was at least as high as when SB was administered in combination with other agents, the intensity of phosphorylation of c-jun was only increased when SB was used in combination with CA and/or the deltanoids (Fig 7). This suggests another level of regulation of c-jun phosphorylation in addition to regulation by JNK kinase activity, perhaps by modulation of the activity of a protein phosphatase.

The principal known function of c-jun is its participation in formation of the AP-1 transcription factor and thereby regulation of the expression of a wide variety of nuclear genes. Since previous work in this laboratory has demonstrated that in 1,25D-treated HL60 cells the AP-1 complex is mainly composed of c-jun and ATF-2 proteins [37], we also examined the levels of phosphorylation ATF-2. While, in contrast to c-jun, no clear relationship of the pattern of ATF-2 phosphorylation to differentiation could be discerned, in several instances there was a reverse level of phosphorylation (one reduced, the other increased) between c-jun and ATF-2 proteins (eg, THP-1 cells in Fig 7), and although SB often increased ATF-2 phosphorylation, CA tended to reduce the SB effect (e.g. U937 cells in Fig 7). Thus, the role of ATF-2 in deltanoid-induced differentiation is not clear at this time, though it is unlikely to have a pro-differentiation activity when phosphorylated.

3.6. Comparison of the effects of 1,25D with JKF on stress-related signaling pathways

In general, there was little difference between the action of 1,25D and JKF on the cell lines studied here regarding the modulation of activating phosphorylations of the stress-related pathways, either when these deltanoids were administered alone, or in combination with differentiation potentiating agents. However, in contrast to 1,25D, JKF consistently reduced the total levels of p54 JNK protein when added to CA/SB-treated cells in all four human cell lines (Fig 7, last lane in relevant panels). Interestingly, this was paralleled by a greater reduction in the level of phosphorylated ATF2, compared to cells which were treated with 1,25D instead of JKF, and was particularly obvious in HL60 cells. This finding raises the possibility that ATF-2 can be targeted for phosphorylation by p54 JNK, rather than p46 JNK which targets c-jun. It also reinforces the notion that the total protein level of a kinase, not just its phosphorylation level, determines its ability to phosphorylate a target, as pointed out above for p38MAPK phosphorylation of K2 (3.4).

3. Discussion

This investigation, designed to test the potential of the low calcemic analog JKF for inclusion in anti-leukemic therapy regimens, also provided several new insights into the mechanisms that participate in the induction of differentiation of myeloid leukemia cells. Utilizing four human cell lines in which differentiation was arrested at different developmental stages, we

found that there is a commonality among these cell subtypes with regard to only slightly lower differentiation-inducing potency of JKF compared to 1,25D, potentiation of their differentiating action by CA and SB, and the participation of the JNK/c-jun pathway in this process. In anticipation of future in vivo animal experiments we also determined that in WEHI-3B D⁺ cells, a murine model of myeloid leukemia, the “DCS cocktail” has similar potency to the “cocktail” in which 1,25D is replaced by JKF.

In this study we found that while the NSE marker demonstrates deltanoid-induced monocytic differentiation of human myeloid cell lines, the principal focus of this study, in a manner similar to CD11b/CD14 (Fig 3), the potentiators affect these reactions in opposing ways, which depend on other components in the DCS “differentiation cocktail”, and vary between the cell lines, making the interpretation of the results difficult. On the cell level, we also noted that the most potent “differentiation cocktail”, consisting of a deltanoid, CA and SB, causes loss of viability in some cells, suggesting that an apoptotic/necrotic pathways may also be triggered by this cocktail (Figs 4, 5).

The most important mechanistic finding documented here is that an activating phosphorylation of c-jun, but not of the functionally related transcription factor ATF-2 [37], can be detected in all cell subtypes studied when the differentiating action of the deltanoid is increased by potentiating agents (Fig 7). While other pathways such as the Erk MAPK pathway are also important for 1,25D-induced differentiation [38,39], the JNK/c-jun pathway seems particularly important for optimization of monocytic differentiation. Other intriguing facets of 1,25D-induced differentiation reported here include identification of activated p46 JNK as the most likely kinase which phosphorylates c-jun in 1,25D-induced differentiation of myeloid cells, while p54 JNK is a candidate for one of the kinases which target ATF-2. We also report for the first time that protein levels of p38 MAPK are reduced by its pharmacological inhibitor SB202190 in all four cell lines (Fig 5), and that the levels of P-ATF-2 are reduced in cell lines other than HL60 when JKF or 1,25D, are added to the CA/SB “combination”, reciprocally with an increase in c-jun phosphorylation (Fig 7). These previously unexplored aspects of signaling merit further extensive investigation, and animal studies are needed to determine if JKF’s previously reported low calcemic effects in vivo are also present when it is used as a component of a “differentiation cocktail”.

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Structures of $1\alpha,25$ -dihydroxyvitamin D_3 and its analog JK-1624F2-2

Fig.1. Chemical structures of $1\alpha,25$ -dihydroxyvitamin D_3 (1,25D) and its analog, JK-1624F2-2 (JKF). Introduction of the D ring 16 unsaturation and fluorines into the side chain of JKF decrease its susceptibility to initiation of degradation by the CYP24 hydroxylase. The 1α -hydroxymethyl group in the A ring serves to reduce the calcemic properties of this compound.

CD11b/CD14 Expression

Fig.2.

Carnosic acid (CA) and SB202190 (SB) increase the deltanoid-induced expression of cell surface differentiation markers in four human myeloid leukemia cell lines. All four human cell lines studied here were treated with indicated compounds for 72 hours, and differentiation markers CD11b and CD14 were determined by flow cytometry. Note that the expression of differentiation markers was increased in all four cell lines treated with deltanoids, or combinations of deltanoids and CA or SB, or a “cocktail” consisting of a deltanoid, CA and SB (DCS). DCS combination was the most potent inducer of CD11b/CD14 expression. Means $\pm$ SD are shown; n = 3.

NSE Reaction

Fig.3.

Effects of CA, SB and a deltanoid, alone or in combination, on the intracellular differentiation marker NSE (non-specific esterase). High proportions of U937 and THP-1 cells express this enzyme in basal state, so microscopic assessment of the reaction was enumerated for both strongly positive (NSE⁺⁺), and for any evidence of positivity (NSE positive). The proportion of NSE positive cells increased when exposed for 72 hours to CA alone, and to a combination of deltanoids and CA, but SB decreased the effect of such treatments. Means $\pm$ SD; n = 3.

Cell Viability

Fig.4.

Supplementation of deltanoid treatment with CA and SB reduce cell viability in all four human cell lines, as determined by light scatter gating using the flow cytometry instrument. The experimental conditions are described for Fig 3.

Fig.5.

Reduced cell viability is due to both necrosis (PI staining) and apoptosis (Annexin V staining) in U937 and NB4 cells treated for 72 h with DCS differentiation cocktail (D= 1,25D or JK5, 10 nM; carnosic acid, 10 uM; SB202190, 5 uM).

Phosphorylation of stress-related MAPK kinases—p38 pathway

Fig.6.

Western blots showing the inhibition of p38MAPK pathway by SB. In all four human myeloid cell lines treated with SB there was reduced or absent phosphorylation of MAPKAPK2, a downstream target of p38MAPK. Although SB inhibited the enzymatic activity of p38MAPK, its phosphorylation was increased by SB in all cell lines, except in THP-1. However, total p38MAPK protein levels were markedly reduced, and although the signal is not apparent in some lanes, it was detected by optical densitometry (OD) of the film. The OD values are shown below each panel as the ratio of the signal of each band to the signal of the corresponding loading control, Crk-L. The phosphorylated form of MAPKAPK2 is the upper band in the close doublet. All four cell lines were treated with the indicated compounds for 72 hours, and proteins in whole cell lysates were subjected to 10% SDS-PAGE gel electrophoresis, transferred to nitrocellulose membranes, and incubated with the indicated antibodies. The membranes were reprobbed for Crk-L protein as a loading control. The blots shown are representative of three similar experiments.

Phosphorylation of stress-related MAPK kinases—JNK pathway

Fig.7.

Western blots showing activation of JNK pathway by SB202190 (SB). This is indicated by the phosphorylation of at least one isoform of JNK protein, and of c-jun, which correlates with the expression of surface differentiation markers. The level of hyperphosphorylated p46 JNK (JNK1) correlates closely with the level of P-c-jun. Experimental details are similar to those described in the legend to Fig 5. The OD values shown in each cell line below phospho-JNK and total JNK panels are the ratios of the signal of hyperphospho-JNK1 (marked with an *) and total JNK1 to the signal of Crk-L, respectively. Other OD values shown below each panel are the ratio of the signal of each single band or double bands to that of Crk-L.