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Mechanisms of endothelial cell protection by blockade of the JAK2 pathway

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Neria F, Caramelo C, Peinado H, González-Pacheco FR, Deudero JJ, de Solis AJ, Fernández-Sánchez R, Peñate S, Cano A, and Ángeles Castilla M. Mechanisms of endothelial cell protection by blockade of the JAK2 pathway. *Am J Physiol Cell Physiol* 292: C1123–C1131, 2007. First published October 11, 2006; doi:10.1152/ajpcell.00548.2005.—Inhibition of the JAK2/STAT pathway has been implicated recently in cytoprotective mechanisms in both vascular smooth muscle cells and astrocytes. The advent of JAK2-specific inhibitors provides a practical tool for the study of this pathway in different cellular types. An interest in finding methods to improve endothelial cell (EC) resistance to injury led us to examine the effect of JAK2/STAT inhibition on EC protection. Furthermore, the signaling pathways involved in JAK2/STAT inhibition-related actions were examined. Our results reveal, for the first time, that blockade of JAK2 with the tyrosine kinase inhibitor AG490 strongly protects cultured EC against cell detachment-dependent death and serum deprivation and increases reseeding efficiency. Confirmation of the specificity of the effects of JAK2 inhibition was attained by finding protective effects on transfection with a dominant negative JAK2. Furthermore, AG490 blocked serum deprivation-induced phosphorylation of JAK2. In terms of mechanism, treatment with AG490 induces several relevant responses, both in monolayer and detached cells. These mechanisms include the following: *1*) Increase and nuclear translocation of the active, dephosphorylated form of β-catenin. In functional terms, this translocation is transcriptionally active, and its protective effect is further supported by the stimulation of EC cytoprotection by transfectionally induced excess of β-catenin. *2*) Increase of platelet endothelial cell adhesion molecule (PECAM)/CD31 levels. *3*) Increase in total and phosphorylated AKT. *4*) Increase in phosphorylated glycogen synthase kinase (GSK)3α/β. The present findings imply potential practical applications of JAK2 inhibition on EC. These applications affect not only EC in the monolayer but also circulating detached cells and involve mechanistic interactions not previously described.

JAK2/STAT; cytoprotection; anoikis; AG490

INDUCTION OF ENDOTHELIAL CELL (EC) resistance to injury is a critical issue in designing tools for vascular therapeutics. Endothelial preservation is important in an extremely ample array of conditions, extending from pathologies in which endothelial injury is the leading mechanism, e.g., malignant hypertension or hemolytic-uremic syndrome (HUS), to arterial hypertension or simply to mechanical endothelial damage as occurs during vascular interventional procedures. Remarkably, a new perspective of interest has been opened with the recognition of the role of endothelial precursors in the repair of the endothelial layer (28).

Numerous studies have dealt with the subject of endothelial protection, basically employing growth factors and stimulating cytoprotective pathways, e.g., phosphatidylinositol 3-kinase (PI3K)/AKT, stress proteins, or MAP kinases (19). The Janus kinase/signal transducers and activators of the transcription (JAK/STAT) pathway transmits information received from extracellular peptide signals through transmembrane receptors directly to target gene promoters in the nucleus, therefore providing a mechanism for transcriptional regulation without identified second messengers. While signaling through the canonical JAK/STAT pathway has been extensively characterized in stimulated immune cells, emerging evidence indicates that STATs also mediate responses to forms of cellular stress as diverse as ischemia/reperfusion, endotoxin, ultraviolet light, or hyperosmolarity (9). Notwithstanding, data are less than homogeneous as to how JAK inhibition affects cell damage in different models of ischemic injury (10, 15, 20). At present, most of the information available on this subject comes from the field of cardiovascular medicine. In this regard, JAK/STAT signaling appears to have an important role in the development of the cardioprotected phenotype associated with ischemic preconditioning (15). On the other hand, double-edged effects have been reported in models of myocardial infarction, i.e., while JAK/STAT activation reduces apoptosis in the myocardial infarction border zone, JAK2/STAT inhibition by AG490 improves the increase of protein phosphatase-1 activity and decreases the levels of p16-phospholamban, which underlie myocardial dysfunction (10). Moreover, the inhibition of the JAK/STAT pathway may result in a significant reduction in the size of devitalized tissue and in the number of cardiomyocytes undergoing apoptosis (20). Furthermore, JAK2/STAT is involved in the anti-apoptotic effect of granulocyte colony-stimulating factor in severely ischemic myocardium and endothelium (13). Collectively, these data indicate that cellular stress activates STATs in ways that can be either detrimental or supportive of cell survival. Recently, a substantial, specific piece of information has been added, by demonstrating that JAK2 tyrosine kinase mediates oxidative stress-induced apoptosis in vascular smooth muscle cells (27) and astrocytes (11). As a consequence, JAK2 inhibition induces cytoprotection against oxidative aggression. In the same line of evidence, oxidized low-density lipoproteins have been recently shown to induce, by means of lipid peroxidation products, tyrosine

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phosphorylation and activation of JAK2, STAT1, and STAT3, therefore providing support to consider JAK2 as an oxidative stress-activated kinase (21). The actual mechanisms involved in these effects have not been sufficiently characterized; furthermore, data on models with possible applications to vascular pathology need to be generated.

To the best of our knowledge, no information has been published regarding the putative role of the JAK2/STAT pathway in cytoprotective or cytotoxic mechanisms on EC. In this sense, two particularly important types of damage are growth factor deprivation and cell detachment-related death. The latter is mainly due to detachment-dependent apoptosis, a phenomenon that has been designated as anoikis. Anoikis is crucial in determining the death of adult EC losing adherence to the monolayer (12), and its inhibition can be attained in only a few conditions. In this regard, previous literature (18) and preliminary results from our laboratory (Castilla MA, unpublished observations) have shown that inhibition of the glycogen synthase kinase (GSK)3β pathway increases the survival of EC against detachment-related cell death. In terms of mechanism, inhibition of GSK3β with LiCl increases β-catenin and favors its nuclear translocation. In these circumstances, β-catenin acts as a survival factor against detachment-related cell death (26, 29). Also, it is known that GSK3β is a regulator of EC proliferation and anoikis (18).

As a part of the interest of our laboratory on the study of endothelial protective mechanisms (4–6), the hypothesis was raised that JAK2/STAT inhibition may have relevant protective effects on cells permanently exposed to stress, of which EC are a paradigm. Preliminary evidence from our laboratory has shown that JAK2/STAT inhibition strongly protects EC against oxidative damage (24). However, no data are available to date on the effects of JAK2/STAT inhibition on other types of injury involving EC. The advent of highly specific JAK2/STAT inhibitors provides a useful tool for examining this pathway (1, 11, 30).

The present study was aimed to analyze the hypothesis that specific JAK2/STAT inhibition induces significant changes in EC viability and to identify the mechanisms involved. Specific attention was dedicated to the study of protection of EC against detachment-related death, i.e., anoikis. A set of mutually related mechanisms with known endothelial protective properties, namely, β-catenin levels and active β-catenin translocation, platelet endothelial cell adhesion molecule-1 (PECAM-1) expression, AKT phosphorylation, and potential apoptotic activators and inhibitors were examined.

MATERIALS AND METHODS

EC Culture

Bovine aorta EC (BAEC) and human umbilical vein EC (HUVEC) were obtained, characterized, and cultured as described (3, 4), in accordance with the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society (6). BAEC were used as the primary experimental source, and HUVEC were employed for complementary confirmatory experiments.

EC Challenge

The use of confluent, monolayer, or detached conditions was a critical point of this investigation. Confluent EC were submitted to either serum deprivation or cell detachment and suspension culture. In all cases, vehicle alone (DMSO <0.1%), AG490 (Calbiochem, Barcelona, Spain), a tyrosine kinase-blocking inhibitor of JAK2 (11, 22, 27) or its control, and the structurally related non-JAK/STAT inhibitor AG82 (Calbiochem, Barcelona, Spain) were added 45 min before the experimental maneuvers were performed. The Src family protein kinase inhibitor PP2 (10 μmol/l, Calbiochem) was also used. Cell viability was assessed by lactate dehydrogenase release (LDH), as described (6). DNA fragmentation (DNA ladder) of EC in agarose gels was used for further characterization of the apoptotic changes, as described (16). Conventional hematoxylin and eosin staining was used to obtain cell images under the different treatments.

Detached Cell Survival and Reseeding Assays

For triggering detachment-related cell death, after pretreatment, BAEC were removed from the culture plates with trypsin-EDTA and resuspended in 1% FBS medium. This small amount of FBS was necessary to avoid additional damage due to serum starvation. Resuspended cells were seeded onto tissue culture plates coated with poly-2-hydroxyethyl methacrylate (Poly-HEMA) (Sigma-Aldrich, Madrid, Spain). An additional system was used as a comparative control in selected experiments, i.e., BAEC were seeded into a spinner flask (Integra Biosciences, Chur, Switzerland) coated with Sigmacote (Sigma-Aldrich). At the times indicated, BAEC were harvested and processed by flow cytometry, as described (6). Both treatments prevented cell adhesion and hence induced anoikis.

For reseeding assay, BAEC cultured in suspension were allowed to grow for 24 h onto Matrigel-coated plates (Matrigel; BD Transduction Laboratories, Madrid, Spain) as described (6). BAEC were fixed, stained with crystal violet, and microphotographed. Cells were counted on 10 randomly chosen high-power fields by two independent observers blinded for the type of specific experiment.

Studies on β-Catenin and PECAM-1

β-Catenin and PECAM-1 (also known as CD31) levels were assessed by Western blot on total and/or nuclear proteins, using specific antibodies (β-catenin, BD Transduction Laboratories; PECAM-1, R&D Systems, Minneapolis, MN). α-Tubulin was used as loading control as described (6).

Further assessment of β-catenin and PECAM-1 was performed by confocal immunofluorescence. For this purpose, cells grown on glass coverslips were fixed with Merckofix (Merck, Barcelona, Spain), permeabilized (0.2% Triton X-100, 15 min), and incubated in PBS with 1% bovine serum albumin. Slides were incubated overnight with the primary antibodies, washed (PBS, 5 min, 3×), and incubated with a specific secondary antibody coupled to FITC (Sigma-Aldrich). For visualization of nuclei, cells were incubated for 45 min with propidium iodide. Confocal images were obtained with a Confocal System TCS SP20 (Leica, Madrid, Spain). Image quantitation was done by means of the program Image J (National Institutes of Health, Bethesda, MD). Ten randomly selected high-power fields were used for each quantitation.

A β-catenin/TCF activity reporter assay was performed to assess β-catenin functionality. For transfections, cells grown to 60–80% confluence were transfected with 20 ng of pTK-Renilla (Promega, Madrid, Spain) and 200 ng of either pTOPFLASH (Top) or pFOPFLASH (Fop) containing multimerized wild-type and mutated Lef1/Tcf binding sites, respectively, fused to a luciferase reporter gene (a gift of H. Clevers; University Hospital, Utrecht, The Netherlands). Transfections (4-set duplicates, 6 h) were performed with lipofectamine (Gibco, Madrid, Spain). BAEC were treated in the presence/absence of AG490 for 24 h, and afterward, luciferase and Renilla activities were measured using the Dual Luciferase Reporter Kit (Promega). When needed, 400 ng of activator plasmid encoding a metabolically stabilized β-catenin S33Y (a gift of A. Ben Ze'ev; Weizmann Institute, Rehovot, Israel) were added as indicated. DNA quantities were normalized with empty pCI-neovector. To analyze

whether the increased β-catenin was active, additional experiments were performed employing a specific antibody against the active form of β-catenin, dephosphorylated on Ser^{47} or Thr^{41} (Upstate, Lake Placid, NY).

Transient Transfections

Different plasmids were transfected in BAEC using a $CaCl_2$-based method. Briefly, BAEC were grown near confluence (80–90%). The optimal transfection was achieved at a $CaCl_2$-to-DNA ratio of 6 mmol/l to 10 μg/ml, respectively; the $CaCl_2$-DNA complex was performed in HBS buffer [21 mmol/l HEPES, 0.14 mol/l NaCl, 5.5 mmol/l D(+)-glucose, 50 mmol/l KCl, 1.32 mol/l Na_2HPO_4 at pH 7.1]. The transfection mix remained for an additional 6 h; then, BAEC were washed with PBS (×2), and growth medium was added until confluence was reached (48 h). Cell challenge was performed as described above. To evaluate transfection efficiency, all assays were also transfected using pEGFP-N1 vector (BD). Transfection efficiency was >50% in all cases.

A wildtype form of JAK2 (pBOS-HAJAK2) and a kinase domain mutant of JAK2 (pBOS-HAdkJAK2) (a gift from Dr. Dwayne L. Barber, Ontario Cancer Institute, Toronto, Canada) were used. For β-catenin transfection, a wildtype form of β-catenin in a pCINeo vector was used (a gift of A. Ben Ze'ev; Weizmann Institute, Rehovot, Israel). In all cases, an empty vector was employed as control.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was employed to further ascertain the specificity of the effects of AG490. EMSA was performed using nuclear proteins (8 μg) and different consensus oligonucleotides (dsDNA, 0.5 ng) labeled with [γ-^{32}P]dATP in binding buffer 1×. Poly(dI-dC) was included as a competitive DNA. After incubation, samples were separated by acrylamide-bisacrylamide gels. Dried gels were exposed to X-Omat films. Commercial oligonucleotides of STAT3 and TFII were used (Santa Cruz Biotechnology, Santa Cruz, CA). Vascular endothelial growth factor (VEGF, R&D Systems) was used as a positive control of STAT3 nuclear binding (31).

Analysis of Protective and Apoptotic Pathways

BAEC protective pathways were analyzed by Western blot, using rabbit polyclonal anti-Bax (Santa Cruz Biotechnology), mouse monoclonal anti-Bcl2 (BD Transduction Laboratories), rabbit polyclonal anti-phospho-AKT (Ser^{473}) and phospho-GSK3α/β ($Ser^{21/9}$) and their respective loading controls, anti-AKT and anti-GSK3β (Cell Signaling Technology, Beverly, MA). Specific antibodies against JAK2 and phospho-JAK2 and against STAT1, STAT3, STAT5, and STAT6 and their respective phosphorylated forms and loading controls were also used (Cell Signaling Technology). Caspase-3 activity was assayed on confluent BAEC with caspase-3 fluorogenic substrate Ac-DEVD-AMC (BD Transduction Laboratories). Activity was expressed as the signal in a luminometer (exciting wavelength 380 nm, and emission wavelength 440 nm).

Statistics

Results are expressed as means ± SE. Unless stated otherwise, each value corresponds to a minimum of five triplicate experiments. Comparisons were done by ANOVA or paired or unpaired Student's *t*-test, when appropriate. Fisher and Scheffé's tests for multiple comparisons were used to determine the *P* value, which was considered significant at <0.05. The statistical analyses were performed with the Windows SPSS 10.0 package (SPSS, Chicago, IL).

RESULTS

For facilitating their visualization, results have been grouped in experiments involving either attached or detached EC.

Experiments on Monolayer, Attached EC

Endothelial cytoprotection by JAK/STAT inhibition. Treatment with the specific JAK2 inhibitor AG490 protected confluent monolayer BAEC against serum deprivation (Fig. 1). This effect was significant from the 10 μmol/l concentration. A similar effect was obtained by incubating confluent HUVEC with AG490 (50 μmol/l) (%relative cell death with respect to 100% control: 69.5 ± 5.7%, $P < 0.02$). The 50 μmol/l concentration was henceforth used for examining specific points.

A first, fundamental issue was to assess the specificity of the effect of AG490 on the JAK/STAT pathway in BAEC. Such specificity was supported by the results of several experiments:

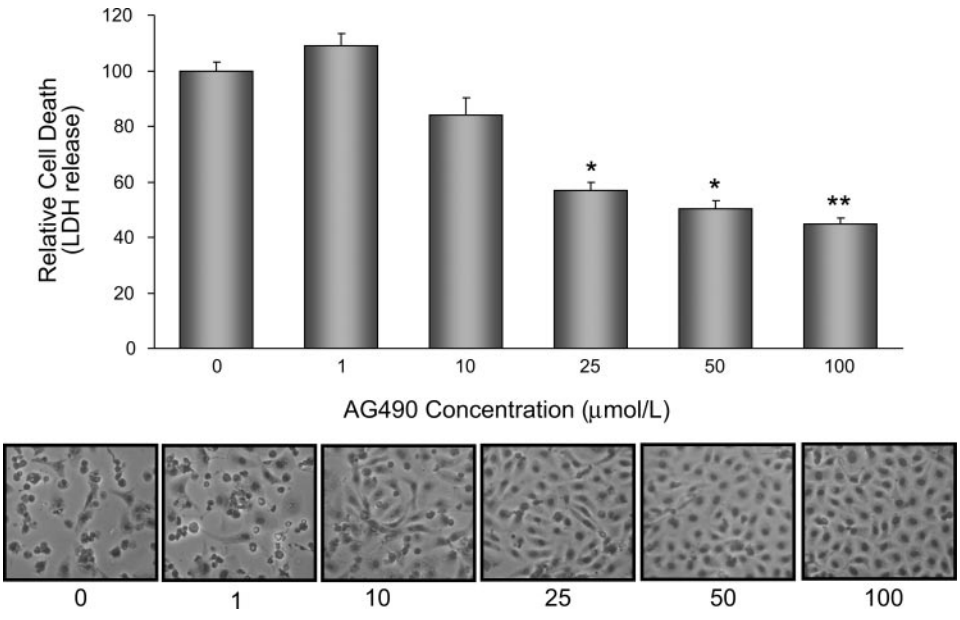

Fig. 1. Cytoprotective effect of AG490 on monolayer endothelial cells (EC). EC damage as induced by FBS depletion on confluent monolayer bovine aorta endothelial cells (BAEC) treated with fresh medium (FM) and FM + AG490 at different concentrations. Cell damage was assessed by lactate dehydrogenase (LDH) release at 48 h (*top*: $*P < 0.05$ and $**P < 0.01$ with respect to the baseline, $n = 6$ triplicate experiments). FM alone was considered as 100% cell death. Phenotypic characteristics of BAEC processed in the same experimental conditions as above are shown at *bottom* (hematoxylin and eosin, ×400). All wells were seeded with the same amount of cells. As can be seen, the integrity of the cell layer increases directly with AG490.

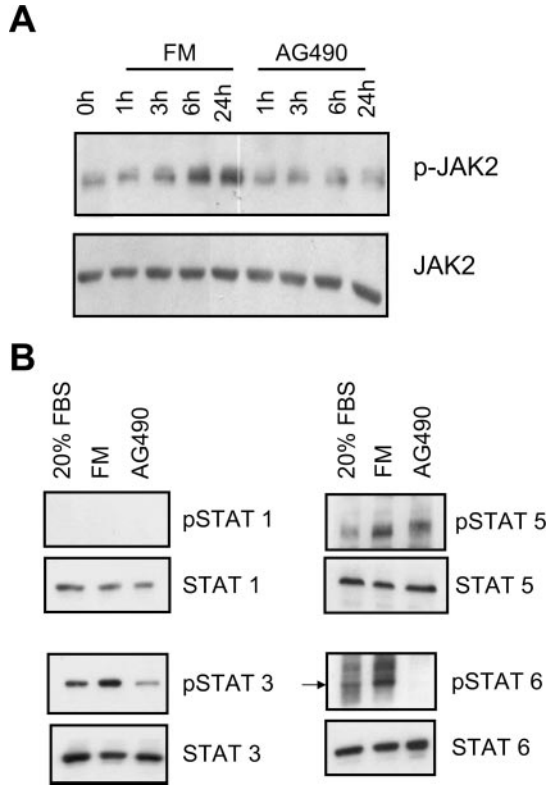

Fig. 2. AG490 effects on JAK2/STAT pathway. *A*: Western blot analysis of phosphorylated JAK2 from total lysates of confluent, monolayer EC, treated with FM or AG490 (50 μmol/l) for times indicated. Total JAK2 was used as loading control. *B*: Western blot analysis of the phosphorylation state of different STAT proteins. Lysates were obtained from confluent monolayer EC treated with FM + 20% FBS, FM alone, or FM + AG490 (50 μmol/l) for 24 h. Respective total STAT antibodies were used as loading control.

1) the inhibition of serum deprivation-induced JAK2 phosphorylation by AG490 (Fig. 2*A*); *2*) the abolition of VEGF-induced STAT3 DNA binding (EMSA) (data not shown); *3*) the inhibition of STAT3 and STAT6 phosphorylation by AG490 (Fig. 2*B*); and *4*) the absence of EC protection by the structurally similar compound AG82, which is devoid of JAK2 inhibitory activity [relative cell death with respect to 100% in control cells: 97.5 ± 2.1%, P = not significant (NS) with respect to control cells]. These results were completed by experiments done in detached conditions (see below).

Changes in β-catenin and PECAM-1. On the basis of previous evidence (2, 29), the hypothesis was raised that treatment with AG490 could act on the levels and distribution of β-catenin. As shown in Fig. 3*A*, AG490 induced a marked increase in total and nuclear β-catenin. The increase of nuclear β-catenin persisted in BAEC pretreated with AG490 and lately cultured in suspension (see below). Additional experiments were done to more specifically assess whether the β-catenin accumulated in the nuclear extract corresponded to the active form. By means of an specific antibody, we found that treatment with AG490 induces nuclear accumulation of the active, dephosphorylated form of β-catenin (Fig. 3*A*, *lane 3*, *left*). When the β-catenin signal was further examined by confocal microscopy, treatment with media +20% FBS induced a marked increase of β-catenin, either at the intercellular junctions, cytosol, or nucleus (Fig. 4*A*). Treatment with AG490 induced changes in β-catenin with a pattern similar to that of 20% FBS, including redistribution to the nucleus. BAEC treated with fresh medium (FM) alone had markedly less amounts of β-catenin, which was located only at the intercellular junctions (Fig. 4*A*, detail). By comparison, the GSK3β inhibitor LiCl favored the localization of β-catenin to the nucleus as well as an increase of lesser magnitude in both cell junctions and cytoplasm (Fig. 4*A*).

At this point, the relevant question was to know whether the increase in nuclear β-catenin under the effect of AG490 was functional. As shown in Fig. 3*B*, AG490 induced a significant increase (1.95 ± 0.2 times, $P < 0.01$) of TOP/FOP activity, comparable with the levels obtained after β-catenin transient transfection (2.28 ± 0.2 times, $P < 0.01$). Additionally, the combination of β-catenin transfection with AG490 treatment

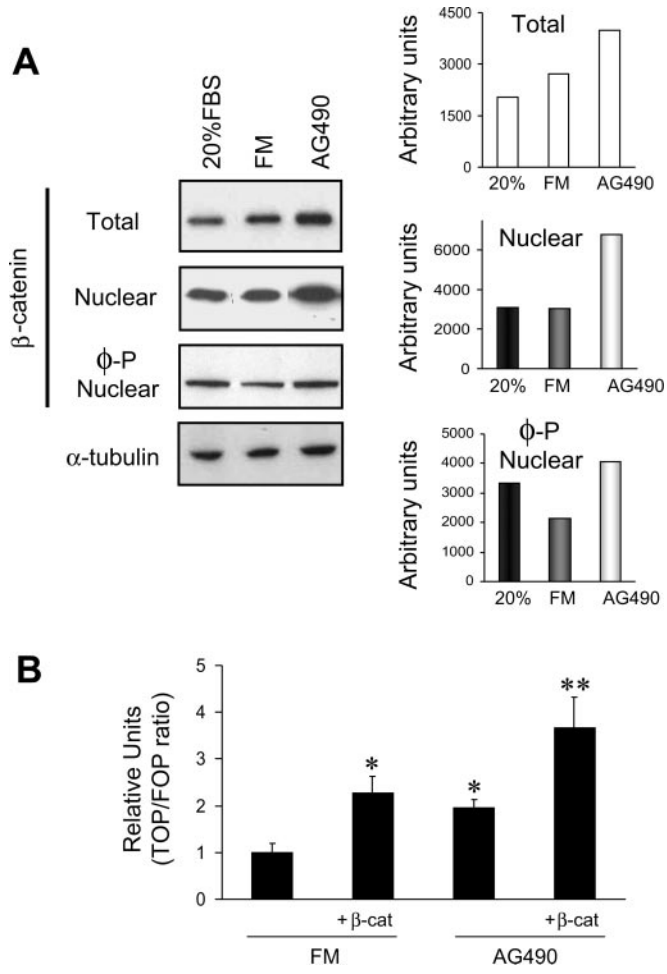

Fig. 3. Changes in total and nuclear β-catenin and β-catenin transcriptional activity under the influence of AG490. *A*: Western blot of nuclear and total β-catenin extracted from BAEC pretreated with FM + 20% FBS, FM alone, or FM + AG490 (50 μmol/l) for 24 h. To further identify the activation of β-catenin, an antibody against active β-catenin, i.e., dephosphorylated on Ser^{37} or Thr^{41}, was also employed, and the intensity of the bands was quantified with respect to α-tubulin and displayed. α-Tubulin was used as loading control. *B*: β-catenin/Tcf activity assay using TOP/FOP luciferase reporter in EC treated with FM or FM + AG490 (50 μmol/l). Bars denoted as +β-cat indicate cotransfection with a plasmid with the β-catenin sequence for overexpression (*$P < 0.05$ with respect to equal condition of FM; **$P < 0.01$ with respect to the first bar, and $P < 0.05$ with respect to second and third bars). ϕ-P, dephosphorylated.

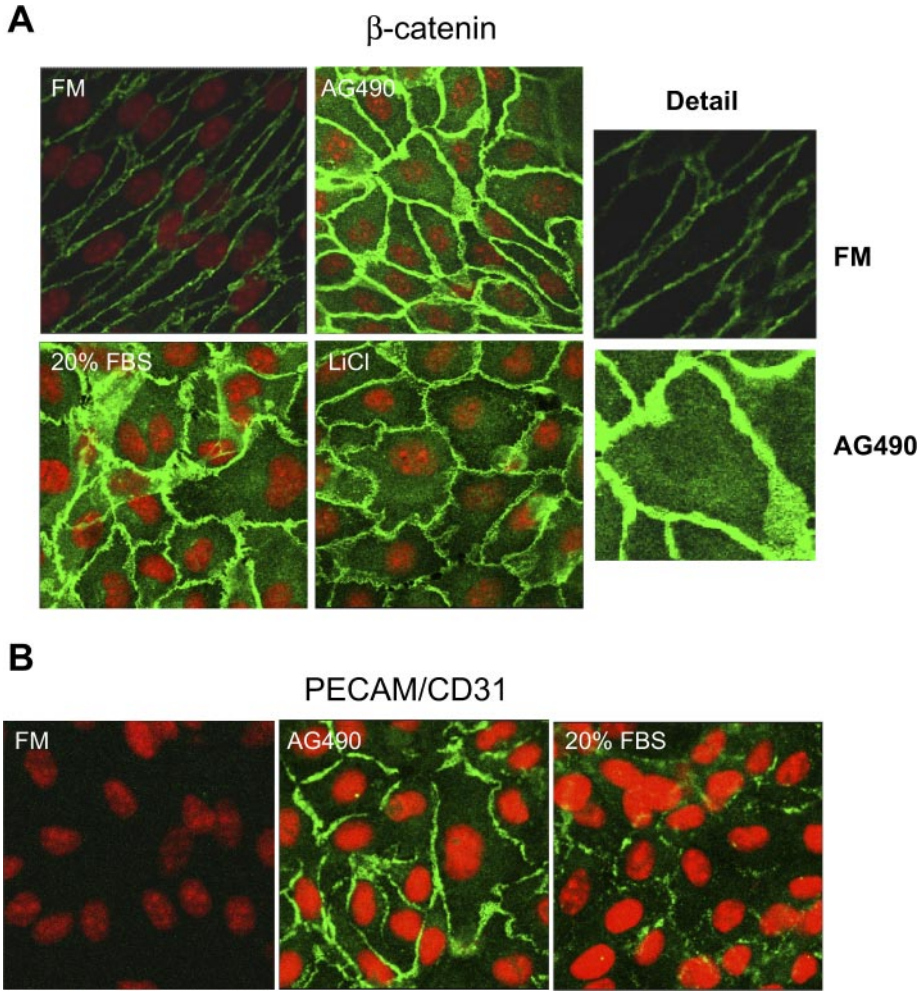

Fig. 4. *A*: immunofluorescence of β-catenin, as visualized by confocal microscopy. Confluent EC grown on glass coverslips were treated with FM, FM + 20% FBS, FM + AG490 (50 μmol/l), or FM + LiCl (20 mmol/l) (positive control) for 24 h. Images show whole cell β-catenin localization with differentiated nuclear contrast (400×). Detailed images of cells treated with either FM or AG490 are shown without using nuclear contrast, for illustrating nuclear translocation of β-catenin. Nuclear staining has been omitted, to improve visualization of the β-catenin *B*: immunofluorescence of platelet endothelial cell adhesion molecule-1 (PECAM-1). The same treatments as in *A* were employed, except for LiCl.

promoted a synergistic activation of TOP/FOP activity. Collectively, all these data indicate a functional role for the translocation of β-catenin observed in this system after AG490 treatment.

A marked increase in PECAM-1 was observed under the same conditions of the β-catenin changes (Fig. 4*B*). This increase was further confirmed by Western blot (data not shown).

Additional Western blot studies were performed to further characterize the effects of JAK2 inhibition by AG490. These studies suggest that, at least in baseline conditions, AG490 may improve BAEC resistance by means of AKT rather than by other anti-apoptotic proteins, as follows. *1*) A significant increase in AKT protein was observed (4.5 ± 0.3-fold increase in AG490 with respect to FM, $P < 0.05$). *2*) No significant changes were found in the Bcl2/Bax ratio (FM 1.00 ± 0.18 vs. AG490 1.19 ± 0.21, $P = 0.07$, $n = 5$). In the same regard, no changes by AG490 were detected on caspase-3 activity (FM 100 ± 8.1 vs. AG490 113.91 ± 17.3 arbitrary units, P = NS).

Experiments in Detached Conditions

Because EC survival is critically dependent on cell-matrix and cell-cell adhesion (23), a set of experiments was performed to assess the role of JAK2 pathway inhibition in cell death related to disrupted adhesion and culture in suspension.

A clear-cut protective effect of JAK2 inhibition was evident on BAEC cultured in suspension. BAEC were pretreated with FM containing no FBS or 20% FBS or FM + AG490 at 50 μmol/l. As shown in Fig. 5*A*, pretreatment with AG490 induced a marked increment in BAEC viability at different times after detachment. Furthermore, inhibition of apoptotic death in detached cells was assessed by examining DNA degradation. As shown in Fig. 5*B*, treatment with AG490 blocked DNA laddering, thus indicating that inhibition of apoptosis was an important component of AG490-induced protection against the effects of detachment.

The specific role of JAK2 inhibition was further ascertained by assessing cell death in BAEC transfected with a mutant dominant negative of the JAK2 kinase domain (a gift of Dr Dwayne L. Barber, Ontario Cancer Institute, Toronto, Canada). As seen in Fig. 5*C*, cell death was significantly inhibited in BAEC transfected with deficient kinase JAK2 against wildtype JAK2 or empty vector. This can be considered a gold standard of JAK2 inhibition and supports the attribution of AG490 effects to JAK2 blockade. From here on, and because of the more direct feasibility of the experiments, the studies on mechanisms have been done mostly

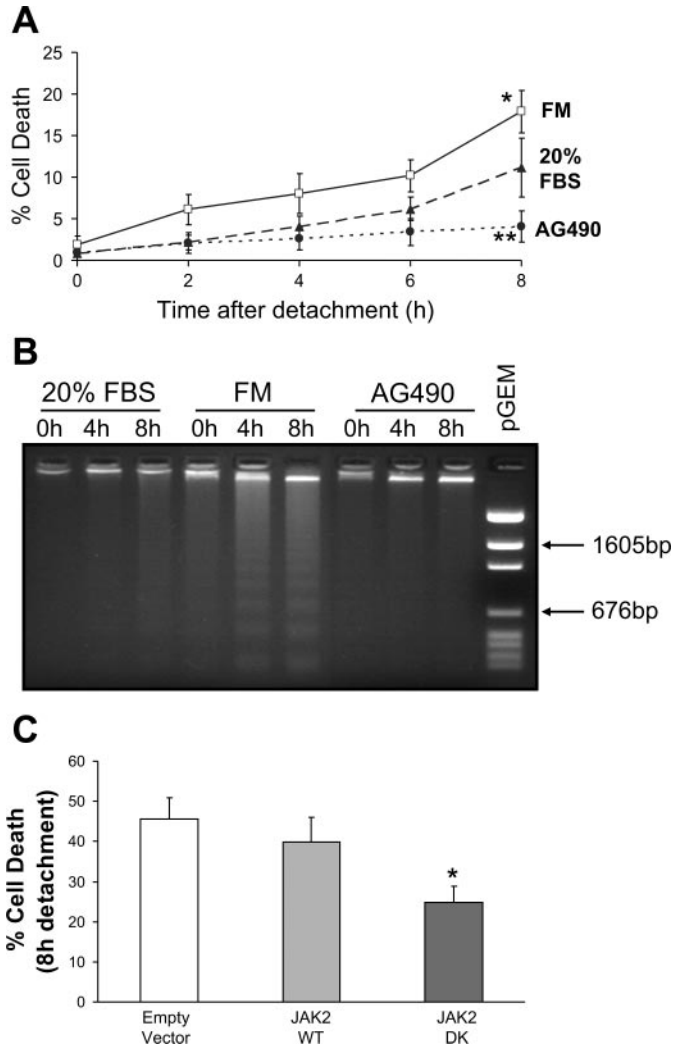

Fig. 5. Protective effect of JAK2 inhibition on EC detachment-induced cell death/anoikis. *A*: cell death progression in EC cultured in suspension after pretreatment (24 h) with FM, FM with 20% FBS, or FM + AG490 (50 μmol/l) as assayed by flow cytometry (*$P < 0.01$ with respect to the 20% FBS and AG490 curves and **$P < 0.02$ with respect to the 8-h point of 20% FBS curve, $n = 5$ triplicate experiments). The curves correspond to 5 experiments run in parallel. In all the samples, the same concentration of vehicle (DMSO, 0.1%) was used. *Time 0h* corresponds to the moment of cell detachment. *B*: DNA ladder from EC, treated as described in Fig. 4*A*. DNA was extracted at indicated times in suspension culture and run in low-melting agarose gel. *C*: cell death analysis measured at 8 h of suspension culture in EC transfected with plasmids encoding a wildtype form of JAK2 (JAK2 WT), a kinase-deficient form of JAK2 (JAK2 DK), or the empty vector (*$P < 0.01$ with respect to the other conditions).

using AG490 rather than JAK2 kinase domain dominant negative transfection.

Because of the putative effects of AG490 on the Src pathway, further experiments were done to analyze the possible participation of Src in the observed protective actions against detachment-related cell death. These experiments showed no effect of the Src inhibitor PP2 on the survival of detached BAEC, as follows: %cell death after 8 h of suspension culture, FM 32.9 ± 5.7 vs. PP2 35.1 ± 6.2 ($n = 4$, P = NS).

The effect of AG490 was not only relevant to BAEC damage, as assessed by flow cytometry, but was also functionally significant in terms of the BAEC growth capability in reseeding (Fig. 6). Pretreatment with AG490 (50 μmol/l) induced a marked increase in the yield of reseeding on Matrigel after culture in suspension (no. of cells 7.8 ± 1.2, 9.4 ± 0.8, and 12.3 ± 1.1 times higher in AG490-treated samples after 8, 24, and 48 h, respectively; all $P < 0.001$ with respect to vehicle-treated controls, $n = 5$, triplicate experiments).

With regard to the mechanisms involved in the increased survival in suspension after JAK2/STAT inhibition, the findings in detached BAEC were in agreement with the results obtained in monolayer BAEC. As shown in Fig. 7*A* (*top*), nuclear β-catenin levels are increased in BAEC pretreated with AG490 and lately cultured in suspension compared with FM or 20% FBS. This result posed the question of whether the increased β-catenin has a significant role in improving BAEC survival in detached conditions. This issue was examined by transfecting BAEC with a plasmid that increases the expression of β-catenin. In these conditions, a marked increase in detached BAEC survival was observed. (Fig. 7*B*), thereby supporting the importance of β-catenin as a major mechanism.

Additional experiments were done to further ascertain the putative mechanisms of the AG490 protective effects in nonadherent cells. As can also be seen in Fig. 7*A*, meaningful effects of AG490 treatment were found on the following potentially protective mechanisms: *1*) higher levels of total AKT; *2*) increased levels of phosphorylated AKT, i.e., activated AKT; and *3*) increased levels of phosphorylated GSK3β, i.e., inactivated GSK3β.

DISCUSSION

Our results provide the first evidence of the potent endothelial cytoprotective effect induced by specific inhibition of the JAK2/STAT pathway. Actually, a singularly important lethal process, i.e., detachment-related cell death, is significantly blocked by pretreatment with the specific JAK2 inhibitor, the tyrphostin AG490, and specifically confirmed by transfection of a JAK2 dominant negative. This effect is accompanied by an increase and redistribution of β-catenin, and by higher levels of total and active phosphorylated forms of a key defensive protein, AKT. Furthermore, the present experiments reveal that a series of mechanisms are activated on JAK2/STAT inhibition.

Although the mechanistic distinction between apoptotic and necrotic forms of cell death is not a central issue of the present investigation, our results, using both DNA laddering and LDH release, indicate that AG490 exerts protective effects on both types of EC elimination. In this regard, the relevant point is that JAK2/STAT inhibition protects BAEC against death triggered by either serum deprivation or detachment.

In BAEC, β-catenin acts both as a vascular endothelial (VE)-cadherin-associated protein in the adherens junction complex and also as a transcriptional activator; the latter role is mediated through its interaction with members of the TCF/Lef family of transcription factors in Wnt signaling (14, 26, 29). In adherent nonstimulated cells, β-catenin is localized to the adhesion complexes; simultaneously, its cytoplasmic levels are kept low, by association with other proteins, e.g., adenomatous poliposis coli (APC) or GSK3β, which drive cytoplasmic β-catenin to proteasome-mediated degradation. The mechanisms identified in the present study are time related to the protective effect of JAK2 inhibition by AG490. Changes in

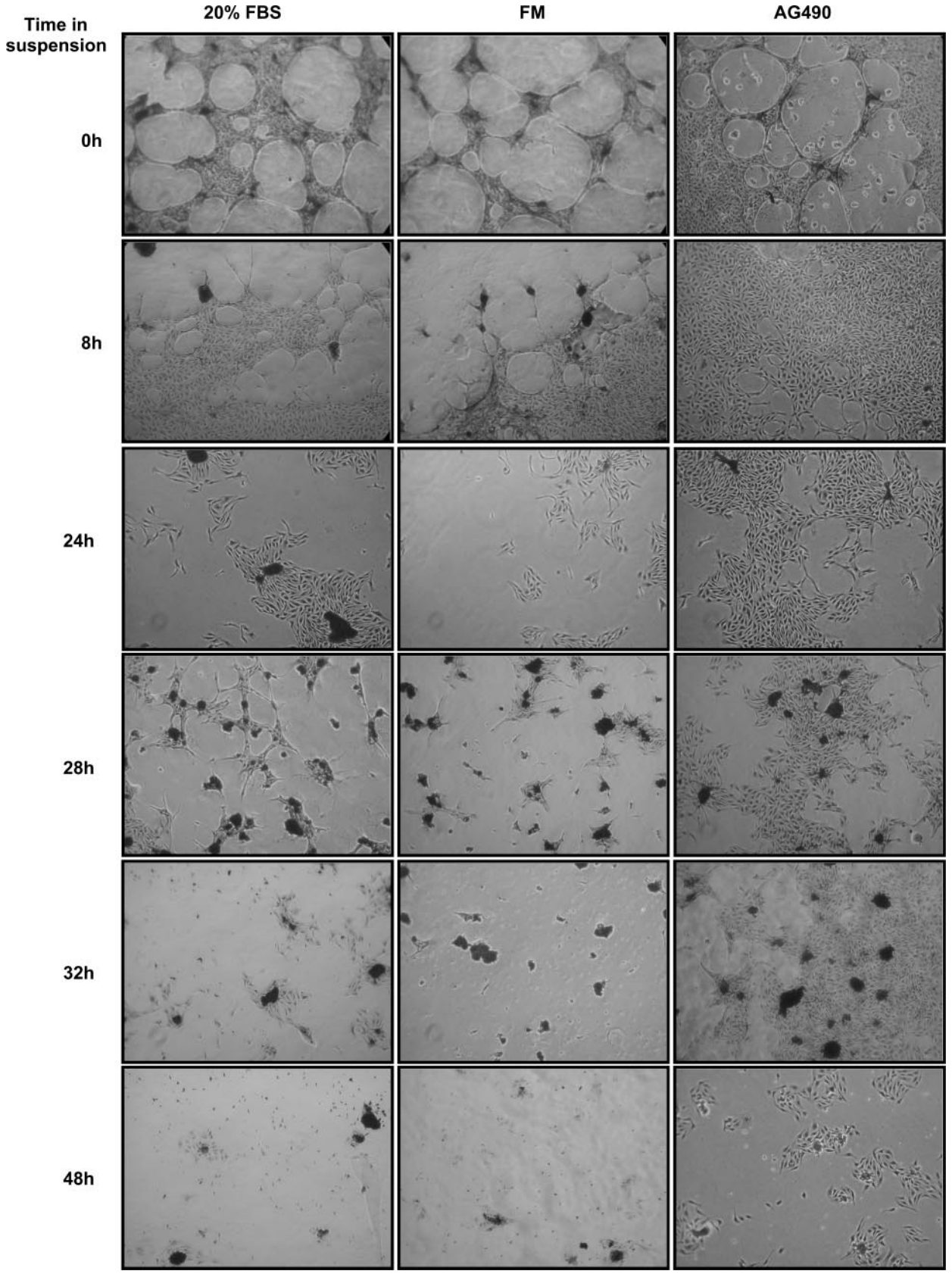

Fig. 6. Effect of AG490 on reseeding efficacy of EC after different times of culture in suspension. EC were pretreated with FM, FM + 20% FBS, or FM + AG490 (50 μmol/l) for 24 h and cultured in suspension for the times indicated, as described in MATERIALS AND METHODS. Thereafter, EC were collected and reseeded on 12-well plates coated with Matrigel. EC were allowed to grow for 24 h, fixed, stained with crystal violet, and microphotographed (×100).

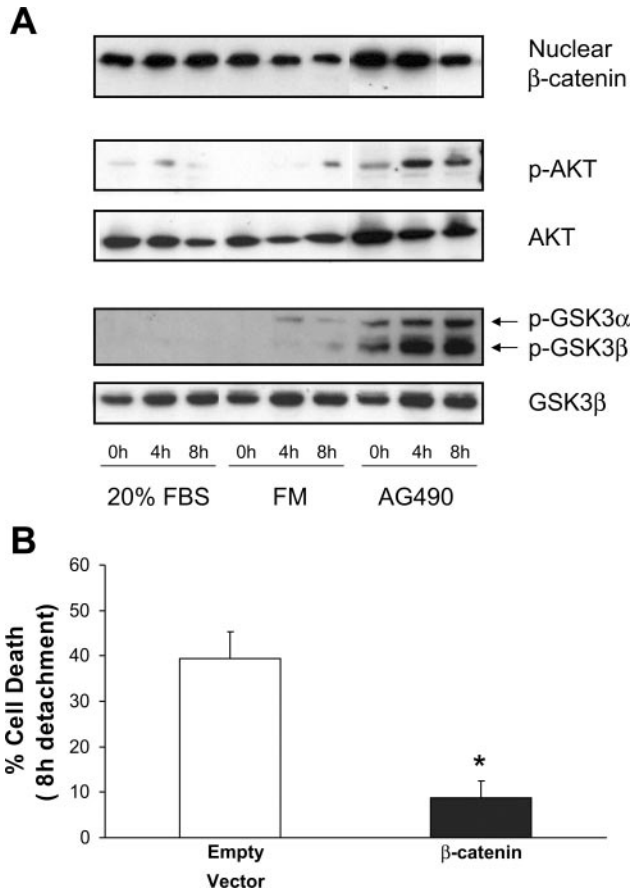

Fig. 7. Influence of β-catenin and phosphatidylinositol 3-kinase (PI3K)/AKT/glycogen synthase kinase (GSK)3 on EC detachment-induced cell death. *A*: Western blot of nuclear β-catenin, phosphorylated AKT and GSK3α/β, and total AKT and GSK3β. Proteins were extracted from cells pretreated with FM, FM + 20% FBS, or FM + AG490 (50 μmol/l) for 24 h and cultured in suspension for the times indicated. The images shown correspond to samples extracted in 1 typical experiment of 5 with similar results and corresponding to the experiments shown in Fig. 4*A*. *B*: cell death measured at 8 h of suspension culture. BAEC were transfected with plasmids encoding a wildtype form of β-catenin or the empty vector (*$P < 0.01$ with respect to the other condition).

quantity and distribution of β-catenin, but especially the demonstration of the nuclear localization of its active dephosphorylated form, and the presence of β-catenin-dependent transcriptional activity support a significant role of this protein in the BAEC protective effects; in fact, the pattern found is coincident with that described in death-resistant hemangioma-derived cell lines (17).

Our results involving PECAM-1 are of importance concerning the β-catenin increase and redistribution to the nucleus. Accordingly, as found by Biswas et al. (2), PECAM-1 is determinately associated with protection from degradation and with increased nuclear β-catenin. In consequence, the simultaneous increase of PECAM-1 and β-catenin accompanying JAK2 inhibition is compatible with the existence of an operational sequence of BAEC protection. Of specific interest, our data go beyond the existing information, all in adherent BAEC, and provide a first piece of evidence on the relationship of β-catenin with the fate of BAEC in suspension. The present results strongly suggest that activation and nuclear translocation of β-catenin can be key elements of survival after detachment.

In resting cells, GSK3β is highly active, and in growth factor deprivation conditions, GSK3β activation is able to increase EC apoptosis (18). Conversely, inhibition of GSK3β signaling, mainly through phosphorylation, protects cells from apoptosis under conditions of mitogen deprivation and regulates cell death by anoikis (18). Inhibition of GSK3β activity results in blockade of β-catenin phosphorylation and subsequent stabilization of cytoplasmic β-catenin, which eventually translocates to the nucleus (8). Our results showing increased levels of phosphorylated GSK3β in BAEC maintained in suspension after pretreatment with AG490 are inversely related to the increase in β-catenin observed in the same circumstances.

Since results showing protective effects of GSK3β inhibition have been previously published (18), we focused our studies only on the comparison of β-catenin patterns of response to LiCl and AG490. In this setting, relevant similarities concerning β-catenin turnover were detected. In fact, the increased phosphorylation of GSK3α and β-catenin increase and translocation under the effect of AG490 are hereby newly described.

Of specific interest, pretreatment of BAEC with AG490 induced a major increase in survival of detached BAEC. This effect was even stronger than that obtained by means of growth factor-rich media, i.e., 20% FBS, and had significant consequences on BAEC reseeding. In fact, no reliable pharmacological tools are available to both prolong life of detached BAEC and enhance their reseeding efficacy, as done by means of AG490. This result can have several practical applications in the field of EC protection.

In terms of mechanism, a strong support for the protective role of β-catenin in the present conditions was provided by the results obtained in β-catenin-transfected BAEC; these cells had a markedly higher survival capability than their empty vector counterparts. To the best of our knowledge, this is the first demonstration that increased β-catenin has a relevant role in prolonging life of BAEC detached from the endothelial monolayer. Furthermore, our results indicate that at least one well-known protective pathway, i.e., PI3K/AKT, is persistently activated in detached conditions under the influence of AG490. The actual functionality of this activation needs further study, by analyzing specific targets of PI3K/AKT activation. On the contrary, the data obtained in the presence of the Src inhibitor, PP2, suggest the absence of a relevant involvement of the Src pathway in BAEC death after detachment and therefore make improbable any possible role of Src inhibition in the effects of AG490. Moreover, it should be noted that the Src inhibitory capability of AG490, albeit in a different experimental system, appears to be rather weak (25).

The present results provide a set of new findings, with relevant potential in terms of practical applications. The strength of the protective effect of JAK2 inhibition against diverse injuries, and particularly against cell detachment-induced death, is particularly valuable.

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