Fludarabine prevents smooth muscle proliferation in vitro and neointimal hyperplasia in vivo through specific inhibition of STAT-1 activation

Daniele Torella,1,2,* Antonio Curcio,1,* Cosimo Gasparri,1* Valentina Galuppo,1
Dania De Serio,1 Francesca C. Surace,1 Anna Lucia Cavaliere,1
Angelo Leone,1 Carmela Coppola,3 Georgina M. Ellison,2 and Ciro Indolfi11Laboratory of Molecular and Cellular Cardiology, Magna Graecia University, Catanzaro, Italy; 2The Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, United Kingdom; and 3Ospedale Civile Immacolata Concezione, Piove Di Sacco, Padova, Italy

Submitted 17 August 2006; accepted in final form 2 February 2007

CURRENTLY, SIROLIMUS AND PACLITAXEL drug-eluting stents (DES) represent the most effective solution for in-stent restenosis (ISR) prevention (16, 28–30). Indeed, restenosis rate after DES deployment is <5% (17). These striking results have generated enormous expectations and clinical demand for DES.

However, it cannot be dismissed that DES have the potential drawback of delaying vascular healing, increasing the late thrombotic risk (18, 27). This, in turn, has prompted the request for the second generation of DES employing new coating systems and drugs.

In the past few years, we and other investigators have partially explained the complex molecular mechanism of vascular smooth muscle cell (VSMC) growth control in the restenosis phenomenon after vascular injury (5, 8–15, 25).

Another crucial signaling cascade regulating VSMC activation upon vascular injury is the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. The JAK/STAT pathway has been primarily recognized as the main mediator of the intracellular signaling of the immune system (7, 23, 24). In cultured rat VSMCs, as well as in the vascular wall, it has been demonstrated that JAK and STAT proteins are activated by different mitogenic stimuli (19, 26). Interestingly, ANG II-induced proliferation of cultured VSMCs is inhibited by either pretreatment with AG490 or electroporation of anti-STAT-1 or anti-STAT-3 antibody, which suggests that STAT-1 and STAT-3 play an essential role in VSMC proliferation in vitro (26). More importantly, AG490, a JAK2-specific inhibitor, reduces VSMC proliferation and prevented neointimal hyperplasia after balloon injury in rats through JAK2-STAT-3 axis inhibition (26). However, the role of STAT-1 inhibition on VSMC proliferation, both in vitro as well as in vivo, remains unknown.

Fludarabine, a purine analog used in the treatment of hematological malignancies, exerts its immunosuppressive activity through the inhibition of STAT-1 signaling (3, 22).

Accordingly, we first investigated the STAT-1 role per se on VSMC growth and the effects of fludarabine on STAT-1 activation and VSMC proliferation in vitro. Local delivery of fludarabine was then employed to prevent neointimal formation in the rat model of vascular injury. Finally, we designed a fludarabine-eluting stent whose efficacy in preventing neointimal hyperplasia was tested in a rabbit stenting model.

METHODS

Cell culture. VSMCs were isolated from the aorta of male Wistar rats weighing ~350–500 g, as previously described (2). For cell culture experiments, 2 × 10^5 rat VSMCs were plated in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). Semiconfluent VSMCs were starved by incubation in 0.5% FBS/DMEM for 36–48 h and then serum-stimulated with normal media containing 10% FBS. Fludarabine was added to the culture media at various concentrations (3, 22).

Cell proliferation. Cell proliferation was assessed at different time-points by measuring DNA content using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (23).

**The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.**
growth medium (i.e., DMEM containing 10% FBS) in the presence or absence of fludarabine (50 μM).

**STAT-1 short hairpin RNA and VSMC growth in vitro.** To establish the specific role of STAT-1 per se on VSMC growth, rat VSMCs were transfected with SureSilencing short hairpin RNA (shRNA) plasmid for rat STAT-1 (SuperArray) by Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. Briefly, plasmid vectors containing the shRNA construct and the green fluorescent protein (GFP) gene were transformed into E. coli cells and subsequently purified (Qiagen). VSMCs were seeded in six-well plates and transfected with STAT-1 shRNA or the negative control shRNA using Lipofectamine 2000. After 48-h incubation, cells were detached, and an aliquot of cells was analyzed by flow-activated cell sorting (FACS) to assess the efficiency of transfection by detecting GFP-positive transfected cells. shRNAs’ plasmid transfected VSMCs were then replated and, after 36 h of starvation in 0.5% FBS/DMEM, were stimulated with 10% FBS/DMEM for 24 h. Bromodeoxyuridine (BrdU) incorporation was measured as described below. Also, cells from separate plates were lysed to verify actual shRNA-induced knockdown of STAT-1 expression by Western blot (see below).

**BrdU proliferation assay.** Incorporation of the thymidine analog BrdU was measured to determine DNA synthesis (2). Starved VSMCs were grown in complete media for 5, 11, 23, and 47 h. BrdU (10 μM) was added every 6 h, and the incubation was continued for ~1 h before cell fixation at the specific time point. After several washes with PBS, cells were fixed with methanol (10% vol/vol for 10 min at 4°C) and stained for BrdU (Roche), according to the manufacturer’s instructions.

**Western blot analysis.** Starved VSMCs were challenged with complete medium for 5–120 min. The cells were washed with PBS, the proteins extracted, and Western blots carried out, as previously described (2). Primary antibodies for the following proteins were employed: JAK2, phospho-JAK2, STAT-1, phospho-STAT-1, STAT-3, phospho-STAT-3, STAT-5, phospho-STAT-5, STAT-6, phospho-STAT-6, Akt, phospho^{ser}Akt, ERK1/2, and phospho-ERK1/2 (Cell Signaling Technology, Beverly, MA).

**Balloon injury and local delivery of fludarabine.** The animals in this study were handled according to the animal welfare regulation of the Magna Graecia University of Catanzaro, and the protocol was approved by the animal use committee of this institution. Fifty Wistar rats weighing 340 ± 40 g (Charles River, Calco, Italy) were anesthetized with an intramuscular injection of 100 mg/kg ketamine (Sigma Chimica, Milan, Italy) and 5 mg/kg xylazine (Sigma Chimica). Angioplasty of the common carotid artery was performed using a balloon embolectomy catheter, as previously described and well validated in our laboratory (8, 9, 12, 14). Fludarabine was dissolved in 30% pluronic F127 gel (Sigma) to the final concentrations of 2.5, 5, 15, or 25 mg/ml. At the time of balloon injury, gel containing fludarabine or vehicle was applied around the middle segment (2 cm in length) of the right injured carotid artery (0.1 ml per 1-cm length of the artery segment, equivalent to 0.5, 1, 3, or 5 mg of total fludarabine locally delivered), as previously described (8, 13). As a control experiment, 200 μl of fludarabine/gel solution (25 mg/ml) were applied around the sham-operated carotid artery. To study the fludarabine toxicity, laboratory studies were performed at baseline and 2 wk after drug local delivery (25 mg/ml). Arterial pressure and heart rate were measured indirectly by a tail-cuff plethysmographic technique (model 50-0002, Harvard Apparatus, South Natick, MA) (13).

**Morphology.** At the time of final experiment (14 days later), the animals were anesthetized, and the carotid arteries were fixed, cut, and stained with hematoxylin-eosin. The cross-sectional areas of external elastic membrane (EEL), internal elastic membrane (IEL), lumen, media, and neointima were measured using a computerized image analysis system, and the ratios between neointima and media were calculated (8, 9, 12, 14).

**Assessment of VSMC proliferation after balloon injury.** To assess local delivery of fludarabine effects on VSMC proliferation, in an additional 15 animals vascular balloon injury was performed as described above, and either fludarabine, at the doses indicated above or saline solution was administered locally. Two days after balloon injury, the arteries were removed, and immunohistochemistry for Ki67 was performed as previously described (14, 32). A proliferation index (PI) was defined as the number of Ki67 positive cell nuclei in the tunica media divided by the total number of cell nuclei and expressed as a percentage.

**JAK2 and STAT-1 phosphorylation in the balloon-injured artery.** Protein samples were extracted from the homogenates of the carotid arteries isolated from an additional 20 rats 2 and 7 days after balloon injury, as described elsewhere (15, 32). Immunoprecipitation and Western blot analysis for JAK2, phospho-JAK2, STAT-1, and phospho-STAT-1 were carried out with the antibodies specified above, as previously described (15, 32).

**Preparation of fludarabine-eluting stents and rabbit stenting model.** Poly-(n)-butyl-methacrylate (0.2 ml) polymer containing fludarabine (1.5 mg) or vehicle was applied on stainless steel ACS Multilink stents (Guidant, Indianapolis, IN), 11 mm in length, as previously described (2). Vehicle (n = 5) and fludarabine-eluting stents (n = 5) were deployed in the right common carotid artery of 10 New Zealand White rabbits (2–2, 5 kg, Charles River), as previously described (1, 20). Bare metal stents (BMS) were deployed in four additional New Zealand White rabbits. The effect of stent implantation on neointimal formation was assessed 28 days later (1, 11, 20).

**Statistical analysis.** All data are shown as means ± SE. Statistical analysis between groups was performed by ANOVA using a SPSS 10.0 program. When a significant overall effect was detected, Tukey’s test was applied to compare single mean values. A P value <0.05 was considered significant.

**RESULTS**

**Fludarabine inhibits VSMC proliferation in vitro.** To investigate VSMC growth in the presence of fludarabine, we first conducted a dose-response curve by culturing VSMCs with 0.05, 0.5, 5, 50, and 500 μM fludarabine or in the absence of the same (control). Cell number in all conditions was assessed every 12 h for 2 days. This analysis showed that 50 μM is the first effective dose of the drug to inhibit cell cycle (data not shown), confirming previous reports (3). Therefore, a more in-depth analysis of cell growth was carried out by measuring BrdU. VSMCs were serum starved and then were stimulated by 10% serum in the presence or absence of Fludara (50 μm).

At 24 h, fludarabine treatment induced a significant decrease of BrdU incorporation compared with controls (Fig. 1A). The biological effect of the drug was reversible, because its removal from the culture medium resumed cell growth after 24 and 48 h, respectively (Fig. 1B).

**STAT-1 knock down by specific shRNA is sufficient to decrease VSMC proliferation.** Before assessing the effects of fludarabine on STAT-1 activation, we needed to demonstrate the specific role of STAT-1 on VSMC growth. To address this issue, we downregulated STAT-1 in rat VSMCs through a specific shRNA (Fig. 1, C–E). FACS analysis showed that the plasmid constructs carrying STAT-1 shRNA or a control inactive shRNA plus the GFP gene were efficiently transfected into VSMCs by lipofectamines (Fig. 1, C and D). Western blot analysis demonstrated a significant reduction of STAT-1 expression in VSMC transfected with the STAT-1 shRNA compared with VSMCs transfected with the control shRNA and the untransfected cells (Fig. 1E). STAT-1 shRNA was target specific, as it did not affect the expression of other related proteins like STAT-3 (Fig. 1E). After 36-h starvation in 0.5% FBS-
FLUDARABINE AND RESTENOSIS

Did not significantly affect the phosphorylation of ERK1/2 and Akt in cultured rat VSMCs (Fig. 2F). These data for the first time correlate fludarabine-induced cellular growth inhibition with the specific molecular switch-off of STAT-1 activation in VSMCs.

Local delivery of fludarabine decreases VSMC proliferation after balloon angioplasty. Ki-67 is preferable to thymidine and proliferating cell nuclear antigen for labeling proliferating cells in vivo (32). Thus we assessed VSMC proliferation by immunostaining for Ki-67 at 2 days after vascular injury (14, 32). In the carotid arteries treated with local delivery of fludarabine, a strikingly lower ratio of Ki-67-positive nuclei to total cells was observed compared with balloon-injured carotid vessels from controls (PI = 17 ± 3%; P < 0.05 vs. fludarabine) 2 days after balloon injury (Fig. 3). This was indicative of a decreased VSMC proliferation in balloon-injured arteries from fludarabine-treated animals.

JAK2 and STAT-1 phosphorylation in the balloon-injured artery. Immunoprecipitation and immunoblotting demonstrated that JAK2 and STAT-1 are barely expressed in the intact rat carotid artery (Fig. 4). In the right balloon-injured arteries of control mice, the phosphorylation activities are means ± SD of 6 measurements. *P < 0.01 vs. base; **P < 0.01 vs. 12 h; †P < 0.01 vs. control VSMCs (open bars). B: fludarabine (solid bars) removal from the culture medium resumed cell growth after an additional 12 and 24 h. Proliferation activities are means ± SD of 6 measurements. †P < 0.01 vs. control VSMCs (open bars). C: representative flow-activated cell sorting (FACS) analysis for VSMCs positive for green fluorescent protein (GFP*) showing the efficient transduction of the plasmid vector carrying signal transducer and activator of transcription (STAT)-1 short hairpin RNA (shRNA) plus the GFP gene. FITC = GFP fluorescence. Phycoerythrin (PE) = negative control antibody. D: the bar graph shows the similar efficiency of transduction of STAT-1 shRNA and control inactive shRNA as measured by FACS for GFP* cells. E: representative Western blot showing effective and specific downregulation of STAT-1 protein by the relative shRNA plasmid (STAT-1 shRNA) compared with untreated cells (Con VSMCs) or cells transfected with a control inactive shRNA (Con shRNA). STAT-3 blot demonstrates that STAT-1 shRNA was specific for STAT-1 protein without affecting STAT-3 expression. Actin was used to normalize protein expression. F: bar graph showing the effects of STAT-1 knock down by STAT-1 shRNA on VSMC growth in vitro. Proliferation activities are means ± SD of 4 measurements. *P < 0.01 vs. base; **P < 0.01 vs. 12 h; †P < 0.01 vs. control VSMCs and control shRNA (open and hatched bars, respectively).

DMEM, VSMCs were stimulated with 10% FBS-DMEM, and BrdU incorporation was measured. Importantly, after 24-h stimulation, STAT-1 shRNA significantly decreased BrdU incorporation in transfected VSMCs compared with control shRNA transfected or untransfected VSMCs (Fig. 1F). These data convincingly show that STAT-1 per se plays a role in normal VSMC growth.

Fludarabine specifically inhibits STAT-1 phosphorylation in cultured VSMCs. As shown in Fig. 2, serum stimulation produced a progressive JAK2 and STAT-1 activation, as demonstrated by the progressive phosphorylation of JAK2 and consequently of STAT-1, which reached its peak at 30 min. No effects were noticed in the amount of the total JAK2 and STAT-1 proteins (Fig. 2, A and B). In contrast, compared with control, fludarabine induced a progressive JAK2 and STAT-1 phosphorylation, whereas it did not change JAK2 activation (Fig. 2, C and D). Fludarabine did not change total levels of the two proteins. To further assess the specificity of fludarabine on STAT-1 phosphorylation, we also measured the activation of other members of the STAT family, namely STAT-3, STAT-5, and STAT-6, in the presence of the drug (Fig. 2E). Interestingly, fludarabine did not significantly affect the phosphorylation of these three STAT proteins (Fig. 2E). Also, fludarabine
animals, expression of these proteins progressively increased from 2 through 7 days. This was associated with an increased phosphorylation of these two proteins compared with intact carotid arteries (Fig. 4). Fludarabine (1.5 mg) significantly prevented STAT-1 phosphorylation at 2 and 7 days and also reduced the increased amount of this protein from 2 to 7 days (Fig. 4). No significant changes were demonstrated in JAK2 phosphorylation at 2 days, but fludarabine inhibited JAK2-increased expression at 7 days (Fig. 4). This suggests that Fludara directly inhibits STAT-1 activation and transcription, while its effect on JAK2 expression is a secondary indirect event.

Local delivery of fludarabine inhibits neointimal hyperplasia after balloon injury. To determine the net effect of fludarabine-induced STAT-1 and VSMC proliferation inhibition in vivo, we studied the effects of perivascular treatment with different doses of fludarabine dissolved in pluronic F127 gel, a sustained release polymer, on neointima formation 14 days after balloon injury. There were no apparent differences in body weight or other systemic conditions between controls and fludarabine-treated rats (data not shown). No blood or renal toxicity was found in the rats treated with the highest dose of fludarabine during the observation period (Table 1). Additionally, locally applied fludarabine showed neither macroscopic nor microscopic changes in the sham-operated arteries over the course of the study (data not shown). Fourteen days after balloon injury, neointima formation was reduced by fludarabine treatment, and the inhibitory effect of fludarabine was directly related to the applied dose (Table 2 and Fig. 5). The neointimal tissue of the injured carotid artery was reduced from 30 to 55% in the rats treated with the different doses of the drug, respectively, compared with the vehicle-treated rats ($P < 0.01$, Table 1 and Fig. 5). The pluronic gel had no effect per se on the neointimal formation and on neointima-to-media ratio at 14 days after vascular injury (data not shown).

Fludarabine-eluting stent inhibits neointimal hyperplasia after arterial stenting in rabbits. On the basis of the positive data of fludarabine effects on VSMC proliferation in vitro and after balloon injury, we sought to assess the feasibility to create a new DES with fludarabine and to evaluate its efficacy on restenosis prevention in an arterial stenting model in rabbits. To this aim, to coat stainless steel stents, we used 0.2 ml/cm of poly-methacrylate (MA) polymer (2). Fludarabine (1.5 mg) was dissolved into this amount of polymer used to create fludarabine-eluting stents. In control rabbits, 28 days after deployment of the MA-coated stents, a significant neointimal hyperplasia and neointima-to-media ratio were observed (5.0 $\pm$ 0.5 mm$^2$ and 2 $\pm$ 0.02, respectively) (Fig. 6). These
values were not different from the neointimal hyperplasia and neointima-to-media ratio values 28 days after deployment of BMS (5.3 ± 0.5 mm² and 2.1 ± 0.01, respectively; P = not significant vs. MA-coated stents). This proved the absence of any direct effect of the used amount of MA coating on VSMC proliferation in vivo. On the other hand, fludarabine-eluting stents reduced the neointima hyperplasia (2 ± 0.6 mm²; P < 0.01 vs. MA-coated stents) and the neointima-to-media ratio (0.9 ± 0.3; P < 0.01 vs. MA-coated stents) compared with MA-coated stents and BMS (Fig. 6).

DISCUSSION

The major findings of the present study are as follows: 1) STAT-1 knock down through specific shRNA is sufficient to reduce VSMC proliferation in vitro; 2) fludarabine specifically inhibits STAT-1 activation without affecting other STAT proteins and consequently diminishes VSMC proliferation in vitro; 3) local delivery of fludarabine reduces vascular STAT-1 activation and expression and affects favorably arterial remodeling, significantly inhibiting neointimal hyperplasia, after experimental balloon angioplasty in rats; and 4) in a rabbit model of arterial stenting, a significant reduction of the neointimal tissue growth is observed after deployment of fludarabine-eluting stents.

ISR is mainly determined by VSMC proliferation and also inflammatory reaction generating neointimal formation (4, 16, 25). In patients with coronary artery disease, sirolimus- and paclitaxel-eluting stents have demonstrated striking positive results for ISR prevention in different trials (16, 28–30). Importantly, DES have been shown lately to be also beneficial (and more effective than brachy therapy) in the treatment of ISR, which has been considered, along with diabetes, as the darkest problems of the restenosis phenomenon (6, 31). These remarkable positive data have generated a high demand for DES in the scenario of the real world clinical practice (27). Indeed, more than 85% of PCI procedures are performed using DES (29, 30). However, the risk of late thrombotic hard

Table 1. Fludarabine effects on blood and renal parameters

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Fludarabine (5 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN, mg/dl</td>
<td>48.2±3.14</td>
<td>45.50±4.22</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.92±0.07</td>
<td>0.86±0.09</td>
</tr>
<tr>
<td>K⁺, mmol/l</td>
<td>4.83±0.25</td>
<td>5.18±0.25</td>
</tr>
<tr>
<td>Na⁺, mmol/l</td>
<td>144.2±2.23</td>
<td>139.2±5.62</td>
</tr>
<tr>
<td>Red blood cells ×10¹², ×mm³</td>
<td>6.382±0.467</td>
<td>6.462±0.375</td>
</tr>
<tr>
<td>Leucocytes, ×mm³</td>
<td>12.220±2.866</td>
<td>13.900±1.129</td>
</tr>
<tr>
<td>Platelets ×10¹³, ×mm³</td>
<td>879±97</td>
<td>797±201</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>13.42±1.81</td>
<td>14.02±0.22</td>
</tr>
</tbody>
</table>

Values are means ± SE. BUN, blood urea nitrogen.
events after DES deployment secondary to the severe impairment has recently become a serious concern in the clinical setting (18, 27). The consequence of these issues has been the need to rapidly develop the second generation of DES by using better coating systems and drugs.

Numerous copolymers have been applied at the surface of metallic stents to serve as a matrix for drug loading. However, we have shown that poly-acrylate coating (the current coating system for DES) has potential toxic cellular effects per se (2). Indeed, polymer coatings by their nature typically induce inflammatory responses and fibrinoid deposits, and polymeric material may degrade over time, bringing the risk of delayed intimal hyperplasia (2, 25). Therefore, the perfect carrier is still to be searched for.

On the other hand, a good candidate agent should be locally delivered (at the site of vascular injury), in adequate concentrations, without toxic effects and over an appropriate period of time to achieve favorable antiproliferative effects.

Fludarabine is a purine analog currently used in the treatment of hematological malignancies (22). Its pharmacological profile has been well investigated, and its potential side effects are highly predictable (22). Importantly, fludarabine has antiproliferative as well as anti-inflammatory properties (22). Therefore, fludarabine might represent a desirable candidate to be used in DES technology, as a valid alternative to rapamycin and paclitaxel.

A recent study described the activation of JAK/STAT pathway simultaneously in medial and neointimal VSMCs after balloon injury in rats (26). Also, local treatment with a JAK2 inhibitor, AG490, reduced STAT-3 phosphorylation and neointimal VSMC replication in the injured arteries (26). Taken together, it is suggested that induction and activation of the

Table 2. Morphological findings 14 days after balloon injury

<table>
<thead>
<tr>
<th></th>
<th>Neointima Area, mm²</th>
<th>Neointima-to-Media Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.179±0.026</td>
<td>1.206±0.312</td>
</tr>
<tr>
<td>Fludarabine (0.5 mg)</td>
<td>0.127±0.02*</td>
<td>0.752±0.173*</td>
</tr>
<tr>
<td>Fludarabine (1.5 mg)</td>
<td>0.108±0.029*</td>
<td>0.725±0.179*</td>
</tr>
<tr>
<td>Fludarabine (3 mg)</td>
<td>0.105±0.02*</td>
<td>0.719±0.222*</td>
</tr>
<tr>
<td>Fludarabine (5 mg)</td>
<td>0.082±0.028*</td>
<td>0.616±0.069*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.01 vs. controls.

![Fig. 5](http://ajpheart.physiology.org/)

Fig. 5. Local delivery of fludarabine inhibits neointimal hyperplasia after balloon injury. A and B: bars representing neointima area (A) and neointima-to-media ratio (B) of common carotid arteries from CON and fludarabine-treated rats 14 days after balloon angioplasty. *P < 0.01 vs. CON. C: representative histological sections stained with hematoxylin eosin of common carotid arteries from CON and fludarabine-treated (Flu) rats 14 days after balloon angioplasty.
JAK2/STAT-3 pathway play a key role in the mechanisms underlying neointima formation after balloon injury via VSMC replication. Incidentally, fludarabine exerts its immunosuppressive activity through the inhibition of STAT-1 signaling (3).

In the present study, we investigated the specific role of STAT-1 per se on VSMC growth, along with the effects of fludarabine on STAT-1 activation and VSMC proliferation. The knock down of STAT-1 by specific shRNA significantly decreased VSMC proliferation without affecting STAT-3, convincingly showing that STAT-1 per se is needed for normal VSMC growth. Also, it seems reasonable to suggest that the role of STAT-1 in VSMC cell cycle is as significant as that of STAT-3.

Fludarabine was able to reduce VSMC growth in vitro, showing nontoxic cytostatic effects. Importantly, fludarabine induced significant reduction of STAT-1 phosphorylation in VSMCs in vitro, whereas it barely affected JAK2 activation. This molecular effect was specific on STAT-1, as fludarabine did not significantly affect the expression and activation of STAT-3, STAT-5, and STAT-6. Also, fludarabine did not influence the activation of Akt and ERK1/2 in cultured rat VSMCs. Thus these data strongly suggest that fludarabine inhibits cell growth in VSMCs by specifically reducing STAT-1 phosphorylation. Nevertheless, these data do not exclude the possibility that other molecular mechanisms may also play a role in the effects of fludarabine on VSMC growth.

STAT-1 is activated by tyrosine phosphorylation mainly through JAK2 (but also by other receptor tyrosine kinases or several nonreceptor oncogenic tyrosine kinases), and this modification serves as a molecular switch that alters its conformation to allow specific binding to DNA (23, 24). Conversely, negative regulators of STAT-1 signaling fall into three groups: phosphatases, suppressor of cytokine signaling proteins, and protein inhibitor of activated STAT protein. Suppressor of cytokine signaling indirectly blocks STAT-1 through JAK2 inhibition, whereas nuclear phosphatases (reducing STAT-1 phosphorylation) and protein inhibitor of activated STAT-1 (reducing STAT-1-DNA binding activity) directly terminate STAT signaling (21). In the present paper, we show that fludarabine treatment reduces STAT-1 phosphorylation without affecting JAK2 activation. It is highly tempting to speculate that fludarabine, once in the cellular compartment, did inhibit STAT-1 phosphorylation through a phosphatase-like activity or enhancing known STAT-1 phosphatases (including Src homology phosphatase-1, Src homology phosphatase-2, CD45, protein tyrosine phosphatase-1B and T cell protein tyrosine phosphatase-3). However, to properly answer this important question, additional investigations are warranted.

The complex mechanism of fludarabine in vivo molecular effects on STAT-1 is further represented by the study of Friedberg et al. (4). In their study, the authors show that fludarabine decreases STAT-1 levels in chronic lymphocytic leukemia (CLL) cells in vitro. However, when administered as oral therapy in CLL patients, fludarabine unexpectedly increases STAT-1 levels in CLL cells after the first cycle of therapy. This somewhat unexpected result was actually explained by raising the possibility that such increased STAT-1 expression upon oral fludarabine could be the effect of a survival advantage for CLL cells that express high levels of STAT-1 (4).

As reported by Seki et al. (26), we also found that, in the balloon-injured arteries, expression and the activation of JAK2 and STAT-1 progressively increased after balloon dilation. Fludarabine (1.5 mg) significantly reduced STAT-1 phosphorylation, and it also prevented the increase of this protein from 2 to 7 days. No significant changes were demonstrated in JAK2

Fig. 6. Fludarabine-eluting stent inhibits neointimal hyperplasia after arterial stenting in rabbits. A: bar graph showing neointimal area and neointimal-to-media ratio 28 days after stent deployment in the three group of rabbits included in the study. *P < 0.01 vs. all. B: representative histological sections stained with hematoxylin/eosin of stented carotid arteries from rabbits 28 days after deployment of bare metal, poly-methacrylate (MA)-coated, and fludarabine-eluting stents.
phosphorylation at 2 days (as demonstrated on VSMCs in vitro), but fludarabine prevented JAK2-increased expression and activation at 7 days. These data suggest that fludarabine directly inhibits STAT-1 activation and transcription, while its effect on JAK2 is a secondary indirect event. How this latter molecular switch takes place remains to be elucidated.

When locally released on the periadventitial surface of balloon-injured rat carotid arteries via pluronic gel, fludarabine significantly prevented neointimal formation without causing any macroscopic or microscopic alteration of various rat organs (data not shown), as well as any blood and renal function alterations.

Thus we produced a custom-made fludarabine-eluting stent using MA as drug polymer carrier (2). Poly-acrylate is an effective polymer of delivering drugs locally and limiting neointima formation in animal models, as well as in human clinical settings (16, 25). However, we and others have shown some undesirable toxic cellular effects of this coating system in vitro and in vivo (2, 16, 25). To circumvent this hazard, we used a dose of MA (0.2 ml per 1-cm stent length) that we have shown to have a minimal pro-apoptotic effect on VSMC in vitro (2). Into this coating, 1.5 mg of fludarabine were dissolved, an average dose proven to be effective in the rat model of restenosis. Importantly, when this “custom-made” new device was tested in the rabbit model of arterial stenting, the fludarabine-eluting stent was specifically efficacious in reducing by ~50% neointimal hyperplasia compared with MA-coated stents and BMS. No difference in the vascular response to stents deployment was observed between MA-coated stents and BMS, demonstrating that the in vivo effects of fludarabine-eluting stents were due to the drug, excluding an effect of the carrier polymer effect per se. The present data are clearly preliminary in their very nature, but may provide new insights into the development of the second generation of DES.

Nevertheless, it has to be pointed out that these results on fludarabine-eluting stents have to be interpreted with extreme caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use. Indeed, for the limitation of the coating technique that we used, we cannot caution when extrapolating them to a clinical use.


