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Atorvastatin-Loaded Hydrogel Affects the Smooth Muscle Cells of Human Veins

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ATV: atorvastatin

tPA: tissue plasminogen activator

PAI-1: Plasminogen inhibitor

MMP: Metalloproteinase

Cx: Connexin

HO-1 : Heme oxygenase

HSMCs : Human Smooth Muscle Cells

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ABSTRACT

Intimal hyperplasia (IH) is the major cause of stenosis of vein grafts. Drugs such as statins prevent stenosis but their systemic administration has limited effects. We developed a hyaluronic acid hydrogel matrix which ensures a controlled release of atorvastatin (ATV) at the site of injury. The release kinetics demonstrated that 100% of ATV was released over 10 h, independently of the loading concentration of the hydrogel. We investigated the effects of such a delivery on primary vascular smooth muscle cells isolated from human veins (HSMCs). ATV decreased HSMCs proliferation, migration and passage across a matrix barrier in a similar dose- (5 -10 µM) and time-dependent manner (24-72 h), whether the drug was directly added to the culture medium or released from the hydrogel. Expression analysis of genes known to be involved in the development of IH, demonstrated that the transcripts of both the gap junction protein Connexin43 (Cx43) and the plasminogen inhibitor PAI-1 were decreased after a 24-48 h exposure to the hydrogel loaded with ATV, whereas the transcripts of the heme oxygenase HO-1 and of the inhibitor of tissue plasminogen activator tPA were increased. At the protein level, Cx43, PAI-1 and the metalloproteinase-9 expression were decreased, whereas HO-1 was upregulated in the presence of ATV. The data demonstrate that the ATV released from a hydrogel has similar effects on HSMCs than the drug freely dissolved in the environment.

INTRODUCTION

Open surgical revascularization is still frequently the best option to treat coronary, lower limbs or cerebrovascular occlusive disease. Nevertheless, restenosis is a major reason of failure in 20 to 50% of these grafts, leading to the partial or complete occlusion of the anastomosis sites (Hwang et al., 2011). Post-vascular intervention stenosis results mainly from intimal hyperplasia (IH) (Hwang et al., 2012; Shah et al., 2003), i.e. the thickening of the tunica intima due to proliferation of vascular smooth muscle cells (VSMCs), and from arterial remodeling, i.e. the rapid alteration of vein grafts implanted into the arterial circulation (Ward et al., 2000). IH is an adaptative process taking place in response to hemodynamic stresses and injuries, and which occurs following bypass graft interventions on arteries, veins and artificial prosthesis (Sugimoto et al., 2009; Zalewski et al., 2002). It is characterized by the hyperproliferation and migration of VSMCs into the sub-intimal region and by an increase in matrix proteins which, together, thicken the tunica intima (Newby, 1997) at the site of injury. This biological cascade is the main trigger of the dedifferentiation of the poorly proliferating, contractile VSMCs into fast proliferating cells secreting extracellular matrix (Alexander and Owens, 2012; Nguyen et al., 2013).

Drugs preventing intraluminal vessel narrowing have been previously identified using an endovascular platform, such as a stent or a plain balloon (Schachner et al., 2006; Wiedemann et al., 2012). However, when open revascularization is mandatory, no platform is available for the local delivery of a drug. Thus, current treatments involve the repeated systemic administration of the active compound, which markedly increases its side effects (Wiedemann et al., 2012). These problems may be decreased by a local application of a drug depot at the site of the surgery. Ideally, such a biocompatible depot

should sustain a controlled and stable release of the active drug for a time sufficient to revert the phenotype of altered VSMCs, and be biodegradable, to avoid the need of a second surgery for its elimination. Here, we have investigated a hyaluronic acid hydrogel matrix, which can be loaded with a variety of drugs known to inhibit the proliferation and migration of vascular smooth muscle cells, as well as the inflammation of the vessel wall (Baek et al., 2012a; Baek et al., 2012b). Among these drugs, the HMG-CoA reductase inhibitors statins, have been shown to be fairly effective in preventing post-surgery stenosis (Qiang et al., 2012). However, the systemic administration of statins has limited effects on this prevention (Stettler et al., 2007), and undesirable side effects have been reported (Taylor et al., 2013)**.** Therefore, we tested the effect of atorvastatin (ATV) after loading in a hyaluronic acid hydrogel on the proliferation, migration and invasiveness of primary smooth muscle cells derived from human saphenous veins. The data document that the hydrogel is a suitable support for the local delivery of drugs, inasmuch as it allowed for the stable release of ATV during hours, which resulted in several modifications of the VSMCs phenotype, alike those induced by the drug freely dissolved in the cell environment.

MATERIAL AND METHODS

Preparation of the atorvastatin-loaded hydrogel

Hyaluronic acid gels (Fortélis extra®), consisting of 25.5 mg/ml crosslinked hyaluronic acid obtained from biofermentation and suspended in phosphate buffer, were generously given by Anteis (Anteis SA Rue de Veyrot 11, 1217 Meyrin, Geneva/Switzerland Meyrin, CH) and were used as received. Calcium atorvastatin (ATV) obtained from Chemos GmbH (Regenstauf, Germany) was dissolved in 33% ethanol aqueous solution and incorporated by gentle stirring. in the hydrogel at the desired concentration. The gel was freeze-dried in a Telstar LyoBeta 15 (Telstar, Terrassa, Spain) using a primary drying at -40°C under 0.2 mBar for 1 h, followed by a 10 h secondary drying at 20°C to eliminate both alcohol and water. It was then reconstituted to the initial volume with sterile MilliQ water over a period of 24 h. This rehydratation restored the macroscopic transparency and viscosity of the unloaded gel, which could be easily disposed with a syringe. For cell culture experiments, the preparation was made under sterile conditions in a laminar flow (Steag LFH07.15, Luftechnik+Metallbau AG, Wettingen, CH), using autoclaved materials.

In vitro release of ATV

The *in vitro* release of ATV was determined under sink conditions. To this end, 100 µl ATV-loaded hydrogel were placed in the 24 wells of a cell culture plate, and covered with a dialysis membrane with a cut off of 14 kDa (Merck, Darmstadt, Germany), which was maintained in place using a silicon O-ring. 500 µl RPMI 1640 (life technologies) culture medium (RPMI) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin were added to each well. At defined time intervals, 200 µl

medium were sampled, and replaced with the same volume of fresh medium. Samples were extracted with 300 µl acetonitrile, and analyzed by HPLC-UV to determine the ATV concentration. The HPLC system consisted of a Waters LC Module 1 (Waters Corporation, Milford, MA, USA) and a Nucleosil, 125/4, 100-5 C_{18} column (Macherey-Nagel, Switzerland). The mobile phase (acetonitrile/10 mM pH 3 acetate buffer: 55/45) was delivered at a flow rate of 1 ml/min. The method has been fully validated and a Limit of Quantification (LOQ value) of 500 ng/ml and a Limit of Detection (LOD value) of 50 ng/ml were obtained. A trueness of 98-102% was determined and the intermediate precision was < 2%; moreover, the three replicates injected at three different days demonstrate the repeatability of the method. The injection volume was 20 µl, and the drug was detected at 245 nm. A standard plot of ATV concentrations ranging from 6.25 to 50 µg/ml was prepared under identical conditions. Release profiles were compared using the similarity factor f_1 and difference factor f_2 (U.S. Food and Drug Administration. Guidance for industry CMC5-1995: immediate release solid oral dosage forms, 1995) (Shah et al., 1998). Equivalent profiles show f_1 value close to 0 (generally less than 15) and f_2 value close to 100 (generally greater than 50). Three independent experiments were run.

Cell culture

Samples of human saphenous veins were obtained from patients undergoing peripheral artery bypass surgery, and prepared for explants culture, as previously described (Corpataux et al., 2005a; Corpataux et al., 2005b). Primary smooth muscle cells were cultured from human saphenous veins from 23 different patients, predominantly male (82%) with a mean age of 68.2 +/- 12,1 years. The Ethical Committee of the University of Lausanne approved the experiments, which are conform with the principles outlined

in the Declaration of Helsinki for use of human tissue. Briefly, vein segments discarded at surgery were placed in RPMI medium. Adhering fat and connective tissue were discarded. The adventitia was carefully removed, and the vessel was opened longitudinally. The inner surface was scraped to remove endothelial cells. Veins explants of 1–2 mm were plated, luminal side down, on the dry surface of a 12-well culture plate. Explants were gently covered with one drop of RPMI medium, and placed overnight in a 37°C incubator gassed with air/5% CO_2. The next day, culture medium was carefully added to the wells, taking care not to detach the explants. Medium was changed every 2-3 days for 1-2 weeks, till cells started to migrate out from the explants. Smooth muscle cells (HSMCs) were identified by immunostaining using antibodies to α-smooth muscle actin (abcam, ab5694) and desmin (Dako, M 0760)**.** Primary proliferating HSMCs, which featured a doubling time of 72-96 h, were grown to confluence, passed once per week, and cultured until passage 3.

Cell proliferation

12500 HSMCs were seeded per well in a 24-well plate and incubated in RPMI 1640 containing 0.4% FCS (growth arrest medium) for 24 h. The next day, the growth arrest medium was removed and replaced with 600µl RPMI 1640 containing 10% FCS supplemented with ATV (5µM or 10 µM). To evaluate the effect of ATV release by hydrogel, only 500 µl RPMI 1640 were added and 100 µl of ATV-loaded hydrogel (5µM or 10 µM) or unloaded hydrogel were placed within a transwell insert of 8 µm pore polycarbonate membrane (Falcon). Cell viability was assessed by the MTT test (Chen et al., 2011; Loo et al., 2011; Wang et al., 2011) prior to the addition of ATV or ATV-loaded gel (time 0), and after 24, 48, and 72 h of culture. To this end, the cells were incubated with 10 µl MTT labeling solution (5 mg/ml) in 200µl medium at 37 °C for 4 h,

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and then solubilized in 200 µl DMSO (Dimethyl sulfoxide). Absorbance at 570 nm was measured with a microtiter plate reader with a reference wavelength of 630 nm, the reaction solvent being used as a blank.

Cell transmigration

The chemotactic-induced transmigration of HSMCs across a matrix barrier was investigated using a Boyden chamber (Back et al., 2005; Corpataux et al., 2005a; Corpataux et al., 2005b; Erices et al., 2011), made of a polycarbonate membrane insert with 8-µm pores-(Falcon) placed in 24-well tissue culture plates. Confluent HSMCs were trypsinized and suspended in RPMI 1640 containing 10% serum supplemented with 0.25% bovine serum albumin and 50 ng/mL Platelet-Derived Growth Factor (PDGF) (migration medium). 10^5 HSMCs in 500 µL migration medium supplemented with various concentrations of ATV (5µM or 10 µM) were loaded into the upper well of each chamber. In the experiments testing the delivering system, 100µl of hydrogel (5µM or 10 µM ATV) deposited on the bottom of wells and covered with a dialysate membrane (Aldrich D9527, MWCO 12 kDa) fixed by a rubber band were preincubated during 24 h in the presence of 500 µL RPMI 1640 which was supplemented with 10% FCS, 0.25% bovine serum albumin and 50 ng/mL PDGF prior to be used for the transmigration experiments. After a 24 h culture at 37°C, the cell suspension was removed from the top of the inserts, which were washed with phosphate-buffered saline (PBS) and fixed in 100% ethanol at -20°C for 30 min. The upper side of the membranes was then rubbed with a moist cotton swab and a spatula to remove the cells which did not transmigrate and the membranes were stained for 10 min with hematoxylin, washed in PBS and examined under a x400 light microscope for scoring the nuclei of migrating

HSMCs. transmigration was expressed as the mean number of migrated cells per high power field.

Cell migration

Cell migration was studied by a wound healing assay (Chen et al., 2011; Erices et al., 2011), using silicon culture inserts (Ibidi®) which define a cell free gap of 500 µm. 15000 HSMCs in 70 µl migration medium were seeded on both sides of the insert. After 24 h, when cells reached a 90% confluence, the inserts were removed and the HSMCs were overlayed with 600µl of culture medium supplemented with different concentrations of ATV. For the experiments involving the delivering system, 100 µl of hydrogel were overlaid in a transwell insert made of a polycarbonate membrane with 8 µm pore-size (Falcon), which was placed in the wells of a 24-well plate, containing 500 µl migration medium. Cultures were photographed at time 0, just after the silicone insert was removed, and thereafter every 12 h for a period of 48 h. Cells that migrated in the 500 µm area initially defined by the silicone insert were counted under a light microscope, at a magnification of x350.

Transcript analysis

The levels of human Connexin43 (Cx43), heme-oxygenase-1 (HO-1), tissue Plasminogen Activator (tPA) and plasminogen activator inhibitor-1(PAI-1) mRNA were determined by quantitative reverse transcription PCR, using the Fast SYBR® Green Master Mix (Applied Biosystems) in a ViiA^{TM}7 Instrument (Applied Biosystems). Briefly, RNA extracted from HSMCs using TriPure isolation reagent (Roche), was treated for 30 min in the presence of DNase I (DNA-free kit, Ambion, Cambridge, UK). One µg total RNA was used for reverse transcription (Promega, Madison, Wisc., USA). Equivalent

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amounts of cDNA from each reaction were processed for RT-PCR analysis. Negative controls included amplification of distilled water, and RNA samples which had not been reverse transcribed. The primers used to amplify specific cDNAs are given in **Table 1**, and were designed using the free online software Primer3 (http://frodo.wi.mit.edu/primer3/) (Alonso et al., 2010a; Alonso et al., 2010b).

Western Blots

HSMCs were washed once with cold PBS and immediately collected and homogenized in lysis buffer containing SDS as published (Alonso et al., 2010a; Alonso et al., 2010b). Protein content was measured using a detergent-compatible DC protein assay kit (Bio-Rad Laboratories, Reinach BL, Switzerland). Samples of total cell extracts (15 µg) were resolved by SDS-PAGE (10 %) and transferred to a PVDF membrane (Immobilon p, Millipore). Membranes were incubated for 1 h in PBS containing 5% milk and 0.1% Tween20 (blocking buffer). Saturated membranes were incubated overnight at 4°C in blocking buffer containing monoclonal anti-Heme Oxygenase 1 antibodies (ab13248 diluted 1/500, Abcam), rabbit polyclonal anti-Cx43 antibodies (AB1728 diluted 1/1000, Millipore), rabbit polyclonal anti-MMP9 antibodies (ab38898 diluted 1/500, Abcam), rabbit polyclonal anti-PAI-1 antibodies (NBP1-19773 diluted 1/1000, Novus Biologicals) or monoclonal antibodies anti-alpha-tubulin (T5168, diluted 1:3000, Sigma-Aldrich). After incubation at room temperature for 1 h with a relevant secondary antibody conjugated to horseradish peroxidase (Fluka Chemie, diluted 1:20,000), membranes were revealed by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Bioscience Europe). Densitometric analyses of immunolabeled proteins were performed using the ImageQuant Software (Molecular Dynamics, Amersham BioscienceEurope).

Immunocytochemistry

HSMCs grown to confluence on glass slides; were fixed for 10 min in acetone at -20°C, air-dried, rinsed in PBS, and permeabilized for 1h in PBS supplemented with 1.5% BSA and 0.1% Triton X-100. The cells were incubated overnight at 4°C in the presence of either a rabbit antibody against Cx43 (3512, 1/100, Cell Signaling), a monoclonal antibody against Heme Oxygenase 1 antibody (ab13248, 1/50, Abcam) or a rabbit antibody against PAI-1 (NBP1-19773, 1/50, Novus Biologicals). Cells were washed and further exposed for 1 h at room temperature to an appropriate Alexa fluor 488 or 594-conjugated antibodies (1/1000; N.V. Invitrogen SA). Cells were then washed, mounted in PBS containing 50% glycerol and 0.4 µg/mL DAPI, and photographed under fluorescence microscopy (Leica Camera AG, Nidau, Switzerland).

Statistical analysis

Data are presented as mean values are given ±SEM. One-way ANOVA was performed to compare the mean values between groups, using the post-hoc Bonferroni test, as provided by the Statistical Package for the Social Science (SPSS 17.0, Chicago, IL). A P value < 0.05 was considered as significant.

RESULTS

Atorvastatin is released from the hydrogel in a controlled manner

The hydrogel released the entire amount of loaded ATV in the cell culture medium over 10 h (**Fig. 1A**) whatever the initial concentration, as indicated by a similarity factor f_2 =62 and a difference factor f_1=5.9. Therefore, the shape of the release curve did not depend on ATV concentration, within the concentration range (2.5-10 µM) studied. In contrast, the release of ATV per hour increased with the initial concentration of the drug (**Fig. 1B**), resulting in significantly different release profiles (f_1<15 and f_2>50 for all pair comparisons). The data show that the release of ATV from the hydrogel is sustained and controlled.

The atorvastatin-loaded gel decreases the viability of HSMCs

The viability of HSMCs increased with time in medium devoided of ATV or containing a drug-free hydrogel, as revealed by the MTT test (**Fig. 2**). A similar pattern was seen when HSMCs were cultured in the presence of 2.5 µM ATV (not shown). In contrast, the addition of 5-10 µM ATV to the culture medium significantly decreased this viability (**Fig. 2**). A similar decrease was observed with HSMCs cultured in the presence of an ATV-loaded hydrogel (**Fig. 2**). This decrease reached significance after 24 h and 72 h culture in the presence of 10 and 5 µM ATV, respectively. From these observations, we choose to test these two concentrations in all further experiments. The results indicate that ATV interferes with the viability of HSMCs, and that this effect is not altered when the drug is released by a hydrogel.

The atorvastatin-loaded hydrogel decreases the transmigration of HSMCs

Control HSMCS rapidly transmigrate across an artificial membrane (**Fig. 3**). A significant decrease in this ability was observed after exposure to 5-10 µM ATV, whether the drug was directly added to medium or was released by a hydrogel (**Fig. 3**). At the same concentrations, ATV, also reduced the migration of HSMCs, as assessed in a wound healing assay (**Fig. 4**). A significant change was observed faster in the presence of 10 µM (24 h) than 5 µM ATV (36 h) (**Fig. 4**). The data document that the drug released by the hydrogel significantly affected the *in vitro* function of HSMCs.

The atorvastatin-loaded hydrogel selectively modulates the expression of markers of HMSCs differentiation

ATV decreased the expression by HSMCs of Connexin43 (Cx43), the metalloproteinase-9 (MMP9) and plasminogen activator inhibitor PAI-1, two markers of IH (Berard et al., 2013; Deglise et al., 2005), at both transcript (**Fig. 5**) and protein levels (**Figs. 6 and 7**). In contrast, the expression of the heme oxygenase HO-1 (Lee et al., 2004) and the tissue plasminogen activator tPA (Berard et al., 2011; Saucy et al., 2010) were up-regulated by ATV (**Figs. 5-7**).These changes were observed whether ATV was directly added to the medium or was released by a hydrogel, but, in the latter condition, became significant with a delay of about 24 h compared to the former condition (**Figs. 5-7**). Immunofluorescence showed that, in spite of these quantitative changes, ATV did not alter the intra-cellular distribution of the Cx43, HO-1 and PAI-1 proteins (**Fig. 7**).

DISCUSSION

The implantation of a vein graft into the arterial circulation often results in the development of intimal hyperplasia (IH), leading to vessel stenosis (Owens, 2010; Owens et al., 2009). This process is associated with the dedifferentiation of HSMCs, which turn from a contractile to a secretory phenotype, characterized by increased proliferation and migration (Alexander and Owens, 2012; Mitra et al., 2006; Nguyen et al., 2013; Owens et al., 2004). Given that IH involves various biological mechanisms, a therapy combining different drugs may help interfering with the vessel stenosis. Candidates, such as statins, have limited effects after systemic administration, due to liver catabolism, digestive clearance and frequent side effects at the high dosage required for systemic efficiency. It would be beneficial to selectively provide the drugs at the site of the stenosis (Wiedemann et al., 2012). As yet, however, no method can achieve such a locally targeted therapy.

As a first approach towards such development, we investigated a hydrogel platform that could ensure a local and controlled delivery of statins in a surgical field. Various thermosensitive gels have been tested for drug administration that, however, usually have a rather short residence time *in vivo* (Le Renard et al., 2010). We have selected a hydrogel made of hyaluronic acid, a key component of extracellular matrix in most native tissues, for two reasons. First, the crosslinking of hyaluronic acid increases the gel viscosity, extending its *in vivo* persistence (Elder et al., 2011). Second, hyaluronic acid could help reduce IH formation (Chajara et al., 2003; Ferns et al., 1995). Here, we have tested such a gel for the delivery of atorvastatin (ATV), a statin which inhibits HSMCs proliferation (Corpataux et al., 2005a; Corpataux et al., 2005b), and IH (Qiang et al., 2012).

Using primary HSMCs from human saphenous veins, we demonstrate that ATV decreases the viability, the proliferation and the transmigration of HSMCs, and that these effects were similarly observed whether the drug was directly added to the culture medium or was loaded on the hyaluronic acid hydrogel. The difference between the two conditions only related to the time course. Thus, the effects of the ATV hydrogel were somewhat delayed compared to those of the free ATV, and were sustained for the 48 h which were investigated here. This time frame should provide a sufficient therapeutic window to interfere with the early steps of IH development, which is launched by the endothelium disruption. The administration of 80 mg ATV results in a maximum plasma concentration of the active molecule of about 50-200 ng/mL after 1-2hours (Bahrami et al., 2005), i.e. concentrations which are significantly lower than those we tested (2.5 - 5 µM) and could explain partially the limited effect of atorvastatin taken orally. The latter concentrations, which were aimed to reach a range of those which are usually tested in vitro (Saijonmaa et al., 2004; Suski et al., 2013) are essential to reach a high local concentration of the drug. The need of elevated concentrations to detect functional effects of ATV in vitro is also likely due the higher proliferation of SMC in culture, which contrast with their usually quiescent state in vivo. Moreover, the continuous stimulation of SMC with the serum, growth factors and nutrients of culture media could participate to the desensitization of cells to lower concentrations of ATV. At any rate, these concentrations differences do not undervalue the interest of our experimental observations, which were all made under rigorously similar conditions for control SMC and cells exposed to ATV.

We also document that ATV differentially regulates the expression of specific genes involved in the development of IH, decreasing Cx43, MMP-9 and PAI-1, and raising tPA and HO-1. By immunofluorescence studies, we further demonstrated that the levels of

Cx43, were time dependently decreased in the presence of both free ATV or ATV release from hydrogel, whereas those of HO-1 and tPA were increased under the very same conditions. Vascular cells express 4 connexins (Cx37, Cx40, Cx43 and Cx45) in various amounts depending on species and vascular beds (Alonso et al., 2010b). These proteins appear involved in different aspects of IH. Thus, we previously demonstrated that Cx43 is expressed in HSMCs of human veins, and is upregulated in the media layer with the development of IH (Deglise et al., 2005). Cx43 participates to control the migration and proliferation of HSMCs (Song et al., 2009), and is increased with the synthetic state of these cells, which develops in early atherosclerotic lesions (Haefliger et al., 2004). Moreover, statins reduced Cx43 in the aortas of atherosclerotic rabbits (Wang et al., 2005). Heme oxygenases (HO) degrade heme to biliverdin, carbon monoxide (CO) and free iron (Otterbein et al., 2000; Otterbein et al., 2003). The exogenous administration of HO-1 reduced the development of atherosclerosis, and restenosis in balloon-injured rat models (Juan et al., 2001; Tulis et al., 2001). Simvastatin increased the cytoprotective HO-1 in HSMCs (Lee et al., 2004), partially accounting for its anti-inflammatory effects (Lee et al., 2004). IH remodeling requires the integrated effects of the fibrinolytic system, the matrix metalloproteinases (MMPs), and their inhibitors. We recently showed that Plasminogen Activator Inhibitor 1 (PAI-1) (Ha et al., 2009) is induced by arterial shear stress, and promotes IH (Berard et al., 2013), likely by enhancing the degradation of the extracellular matrix, which facilitates the migration of HSMCs from the media to the intima layer (Muto et al., 2012). It is remarkable that several of the factors which contribute to IH, are simultaneously but differentially regulated by ATV, in a way that interferes with the pathological remodeling of the vascular wall.

Would the drug, and other candidate statins, be made selectively available at the site of venous stenosis, it may be feasible to prevent or hinder IH, if the effective drug levels can be maintained for the few early days during which the phenotypic change of HSMCs is launched. Our experiments indicate that this can be achieved, at least *in vitro*, using a hydrogel of native hyaluronic acid, which releases active ATV in a sustained way. The results open the way towards the generation of further platforms that could release a combination of anti-stenosis drugs, *in vivo* and over extended periods of time.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Céline Dubuis, Laurence May, Florian Alonso, Ludmila Luca, Ioanna Mylonaki, Florence Delie, Olivier Jordan, François Saucy, Jacques-Antoine Haefliger

Conducted Experiment: Florence Delie, Olivier Jordan, Sébastien Déglise, Jean-Marc Corpataux, François Saucy, Jacques-Antoine Haefliger

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Wrote or contributed to the writing of the manuscript : Paolo Meda, Florence Delie, Olivier Jordan, François Saucy, Jacques-Antoine Haefliger

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FOOTNOTES

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FIGURES LEGENDS

Figure 1: Atorvastatin is released by the hydrogel in the cell culture medium

Upper panel: The kinetic of ATV release is independent of the initial loading concentration. **Lower panel**: In contrast, the amount of ATV released per h increased with the concentration of the drug. Mean values are given ± SEM (n=3).

Figure 2: The atorvastatin-loaded hydrogel decreased the viability of HSMCs

The viability of HSMCs, as assessed by the MTT test, was significantly reduced by 5-10 µM ATV, whether the drug was added directly to the culture medium (Medium) or was released by a hydrogel (Gel). This change was faster (24h) in the presence of 10 µM than 5 µM ATV (72h) *P < 0.05, versus the respective cells incubated in absence of ATV (control).

Figure 3: The atorvastatin-loaded hydrogel decreased the transmigration of HSMCs

Upper panel: Transmigration of HSMCs in presence or absence of of ATV was assessed with the modified Boyden chamber technique.

Lower panel: Quantitative assessment of the transmigrated cells demonstrate a significant decrease in the capacity of HSMCs to transmigrate in presence of ATV in medium or released from the hydrogel. *P < 0.05, **P < 0.01 and ***P < 0.001 versus control cells incubated in absence of ATV. Bar represents 20 µm.

Figure 4: The atorvastatin-loaded hydrogel decreased the migration of HSMCs

Upper panel: Primary HSMCs migration was assessed using silicon culture inserts defining a cell free gap ~500 µm (lines). HSMCs grown under control conditions (control medium) migrated into this gap to fill it in about 48h. This migration was decreased by 5 and 10 µM ATV, whether the drug was added directly to the medium (ATV medium) or was released by the hydrogel (ATV gel). Bar represents 80 µm.

Lower panel: Inhibition of cell migration was observed at 36 h in the presence of 5 µM ATV, and already at 24 h in the presence of 10 µM ATV directly added to the culture medium (left panel). A similar inhibition of HSMCs migration was observed using the ATV-loaded hydrogel (right panel). *P < 0.05, **P < 0.01, ***P < 0.001 versus control cells incubated in the absence of ATV.

Figure 5: Atorvastatin regulates the levels of selective transcripts of HSMCs

After a 24h exposure to ATV directly added to the medium, the expression of PAI-1 and Cx43 mRNA was decreased, whereas that of tPA and HO-1 transcripts was increased. Twenty four hours later, similar changes were detected in HSMCs exposed to ATV released from the hydrogel. *P < 0.05, **P < 0.01 and ***P < 0.001 versus cells incubated in absence of ATV (Control).

Figure 6: Atorvastatin regulates the levels of selective proteins of HSMCs

HSMCs cultured for 24 h in a medium supplemented with ATV showed decreased expression of the PAI-1, MMP9 and Cx43 proteins, but increased levels of the HO-1 protein. Similar changes were observed 24 h later, in cells exposed to an ATV-loaded

hydrogel. *P < 0.05, **P < 0.01 and ***P < 0.001 versus cells cultured in the absence of ATV (Control).

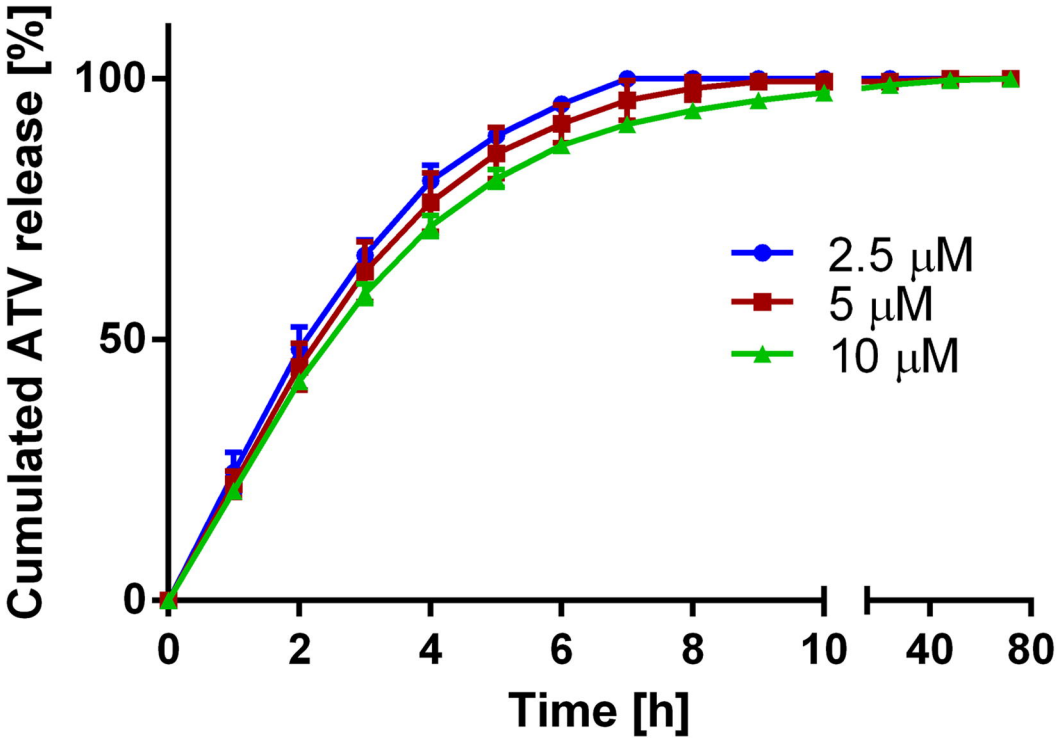
Figure 7: ATV did not perturb the intracellular localization of the proteins it quantitatively regulated

Immunocytochemistry confirmed the decreased expression of Cx43 and PAI-1, and the increased expression of HO-1 in HSMCs exposed to ATV and further revealed that these changes did not affect the distribution of these proteins. Thus, in both control and ATV-treated cells, Cx43 was mostly distributed at the cell membrane, whereas PAI-1 and HO-1 were largely observed within the cytoplasm.

Table 1: Human primers for quantitative real-time PCR

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
PAI-1	GGCTGGTGCTGGTGAATG	ATCGGGCGTGGTGAACTC
Cx43	GAACTCAAGGTTGCCCAAAC	TTAGAGATGGTGCTTCCC
HO-1	AGGAGGTCATCCCCTACACA	GGGGTAGAGCTGCTTGAACT
tPA	ACACAGCACAGAACCCCAGT	CAGGAGGGCACATCACAGTA
GAPDH	AACTTTGGTATCGTGGAAGG	CAGTAGAGGCAGGGATGATGT

Figure 1


Cumulated ATV release [%]
100
50
0
0
2
4
6
8
10
40
80
Time [h]
2.5 μM
5 μM
10 μM

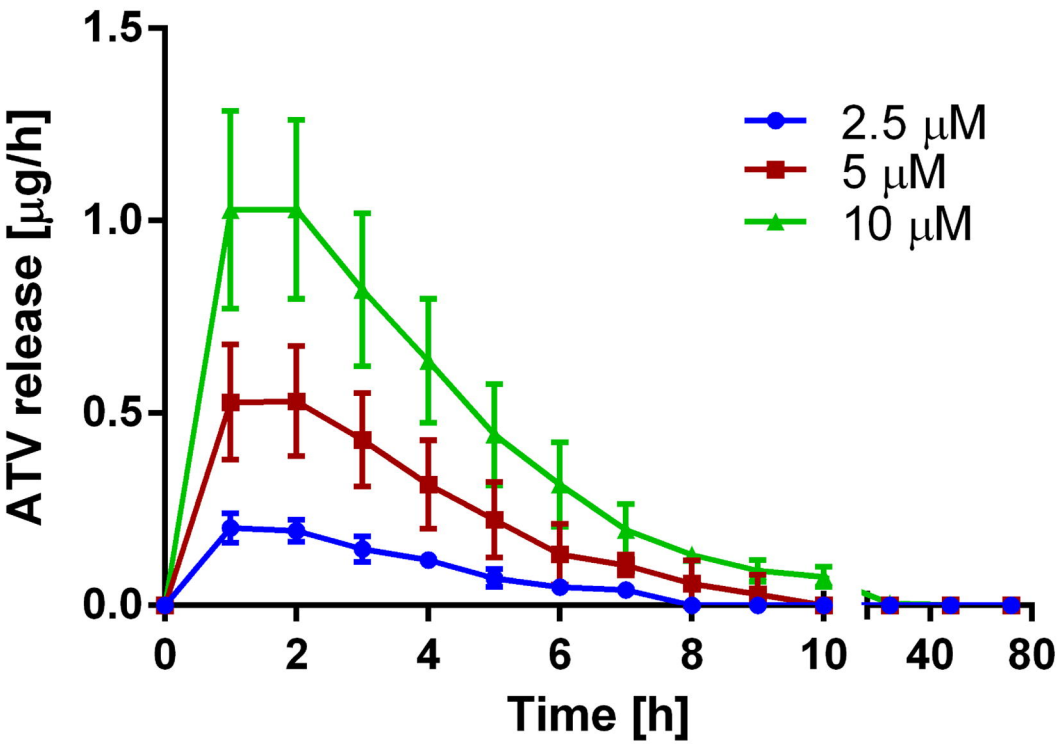

ATV release [μg/h]
1.5
1.0
0.5
0.0
0
2
4
6
8
10
40
80
Time [h]
2.5 μM
5 μM
10 μM

Figure 2

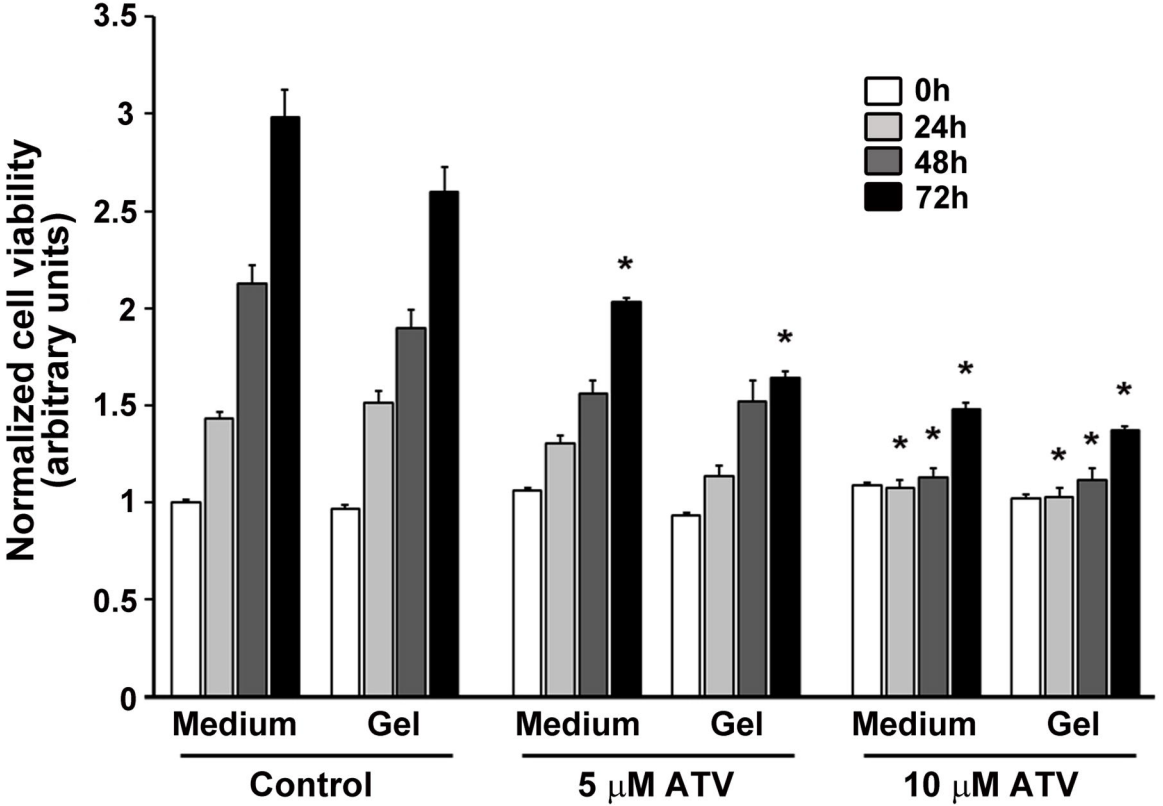
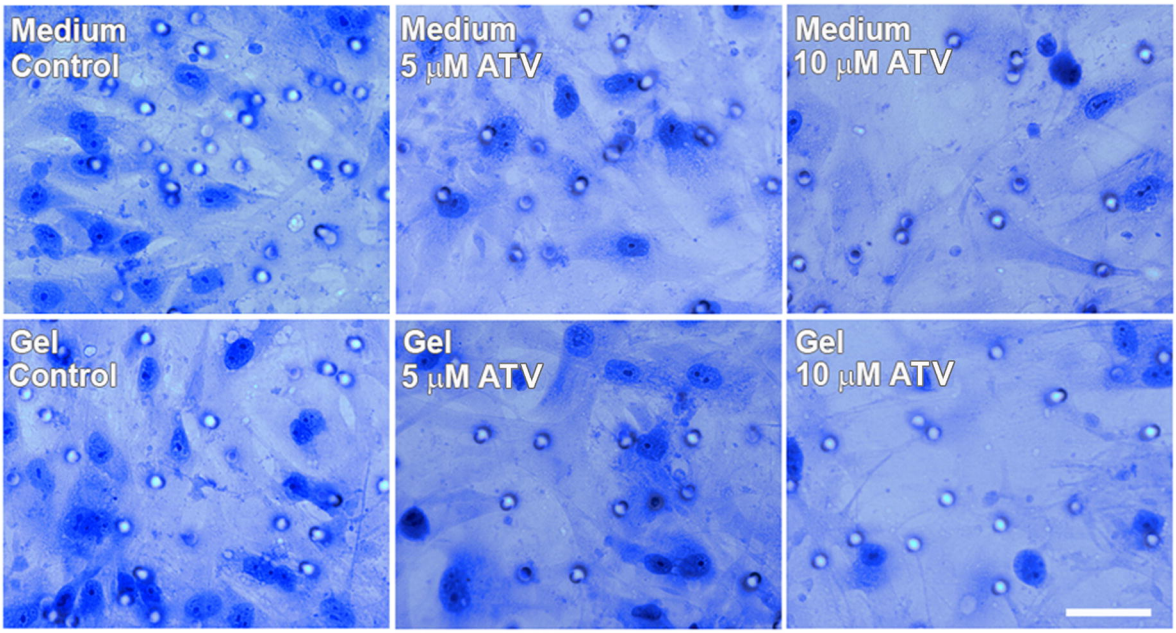

Normalized cell viability (arbitrary units)
3.5
3
2.5
2
1.5
1
0.5
0
0h
24h
48h
72h
*
Medium
Gel
Medium
Gel
Medium
Gel
Control
5 μM ATV
10 μM ATV

Figure 3


Medium
Control
Medium
5 μM ATV
Medium
10 μM ATV
Gel
Control
Gel
5 μM ATV
Gel
10 μM ATV

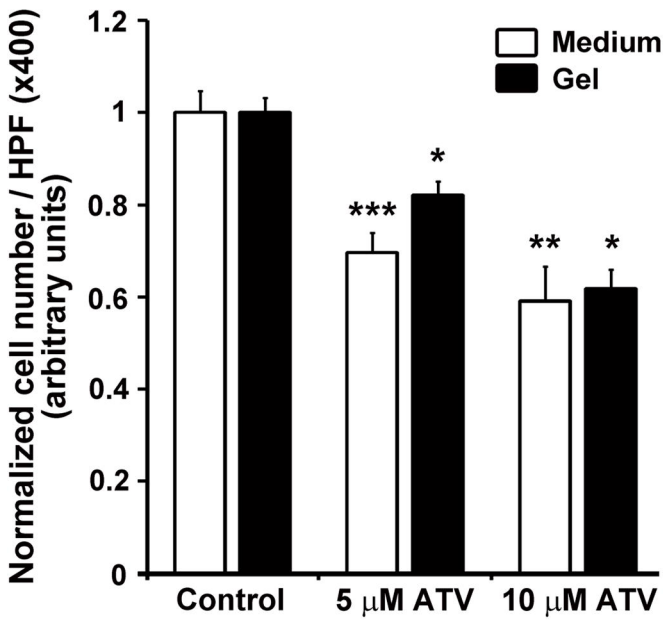
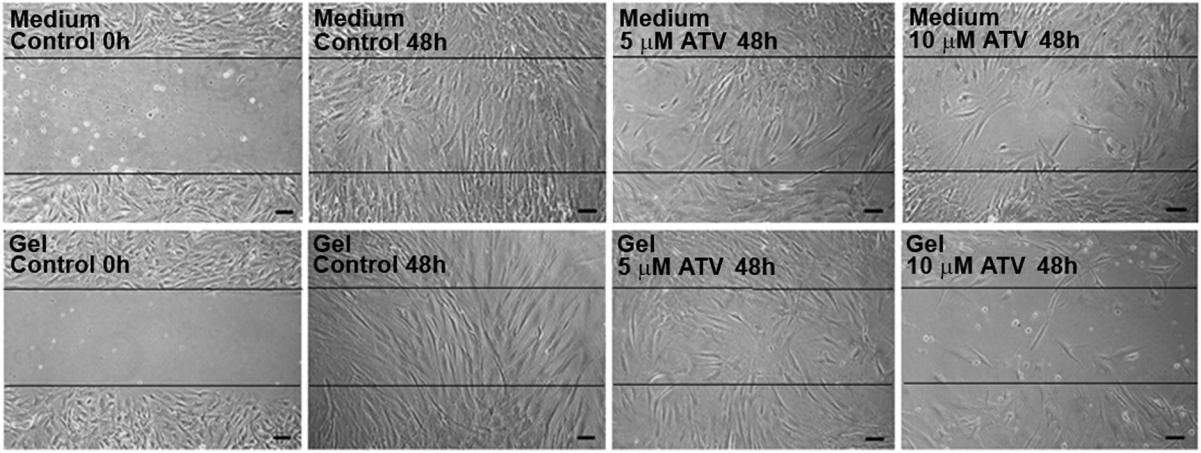

Normalized cell number / HPF (x400)
(arbitrary units)
1.2
1
0.8
0.6
0.4
0.2
0
Medium
Gel
***
*
**
*
Control
5 μM ATV
10 μM ATV

Figure 4


Medium
Control 0h
Medium
Control 48h
Medium
5 μM ATV 48h
Medium
10 μM ATV 48h
Gel
Control 0h
Gel
Control 48h
Gel
5 μM ATV 48h
Gel
10 μM ATV 48h

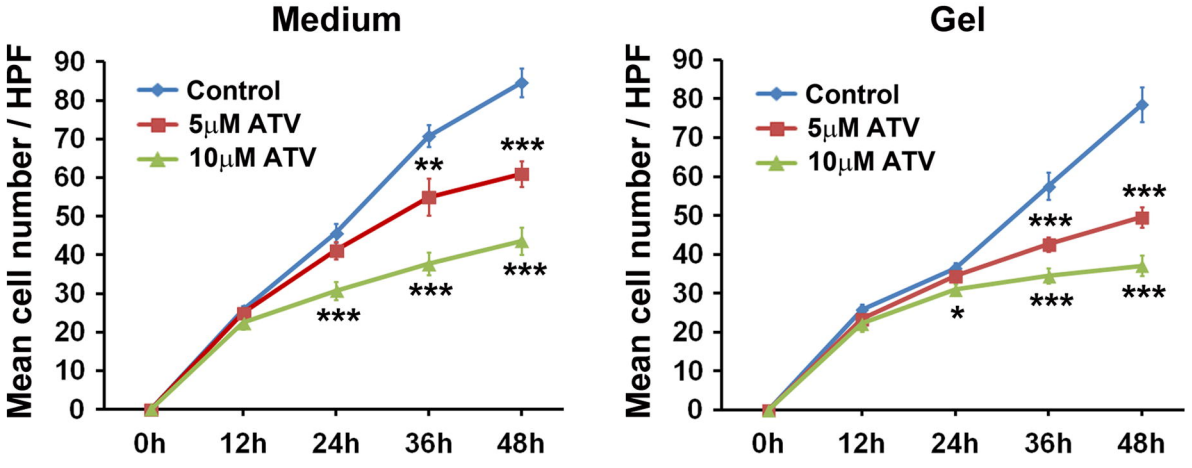

Medium
Mean cell number / HPF
Control
5μM ATV
10μM ATV
0
10
20
30
40
50
60
70
80
90
0h
12h
24h
36h
48h
**
***
***
***
***
Gel
Mean cell number / HPF
Control
5μM ATV
10μM ATV
0
10
20
30
40
50
60
70
80
90
0h
12h
24h
36h
48h
*
***
***
***
***

Figure 5

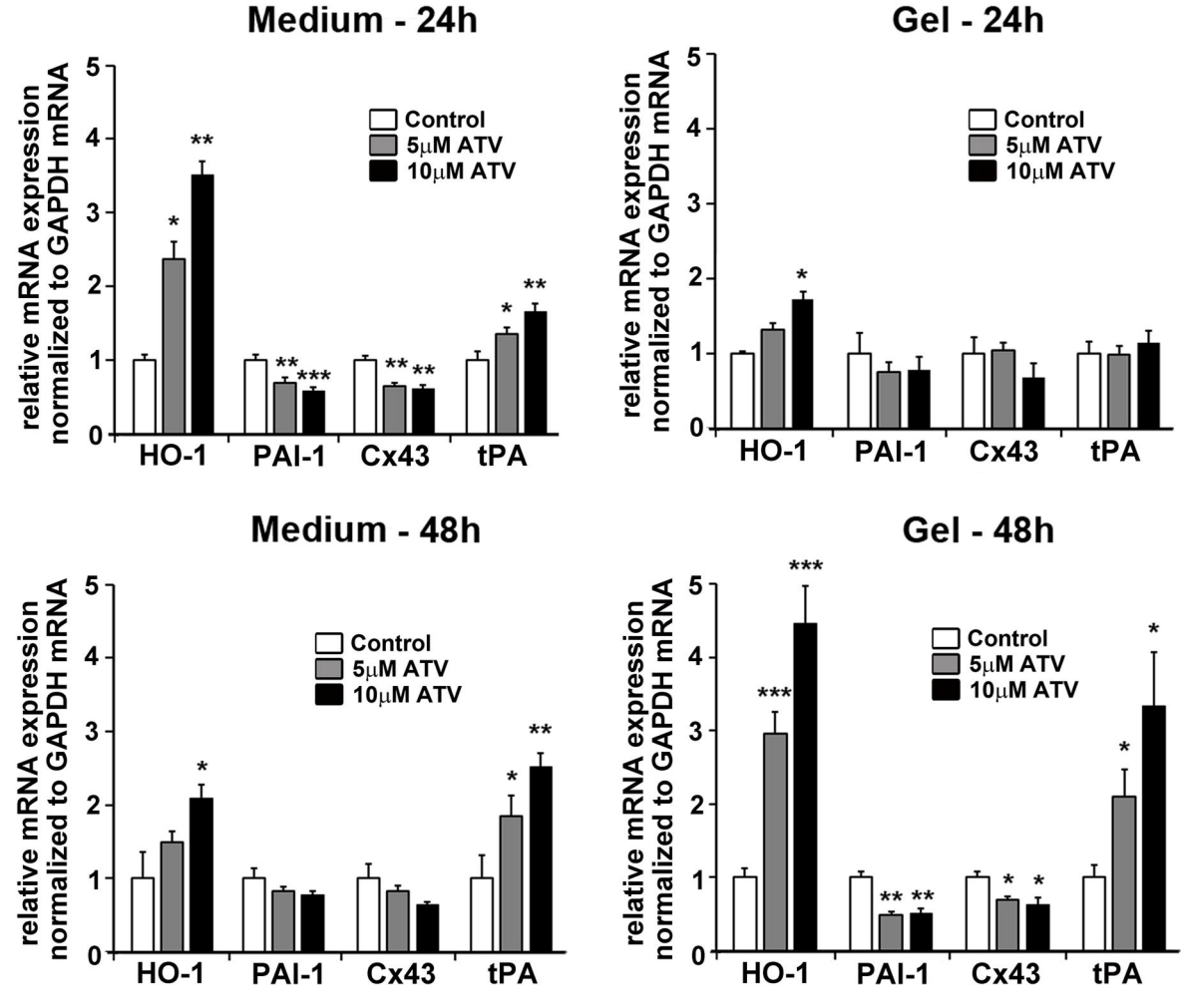
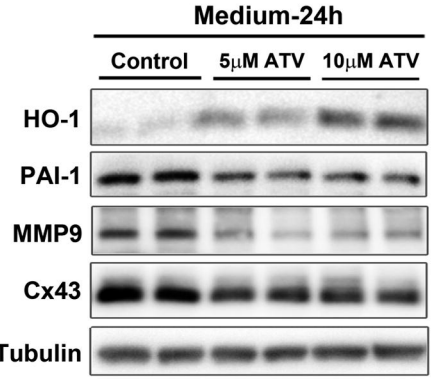
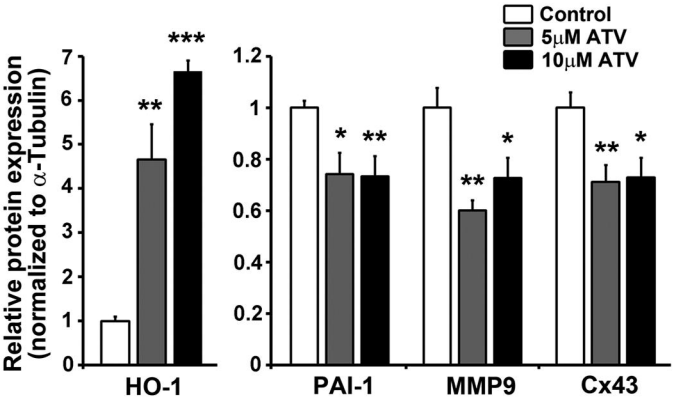
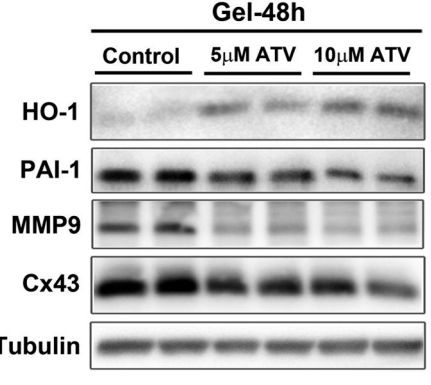

Medium - 24h
Gel - 24h
Medium - 48h
Gel - 48h
relative mRNA expression normalized to GAPDH mRNA
Control
5μM ATV
10μM ATV
HO-1
PAI-1
Cx43
tPA

Figure 6


Medium-24h
Control
5μM ATV
10μM ATV
HO-1
PAI-1
MMP9
Cx43
α-Tubulin


Control
5μM ATV
10μM ATV
Relative protein expression
(normalized to α-Tubulin)
HO-1
PAI-1
MMP9
Cx43


Gel-48h
Control
5μM ATV
10μM ATV
HO-1
PAI-1
MMP9
Cx43
α-Tubulin

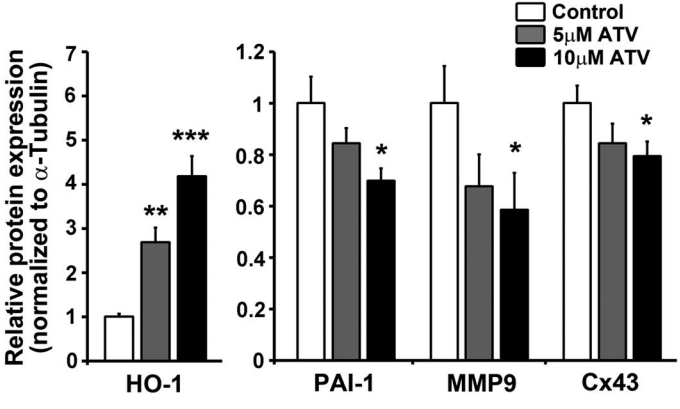
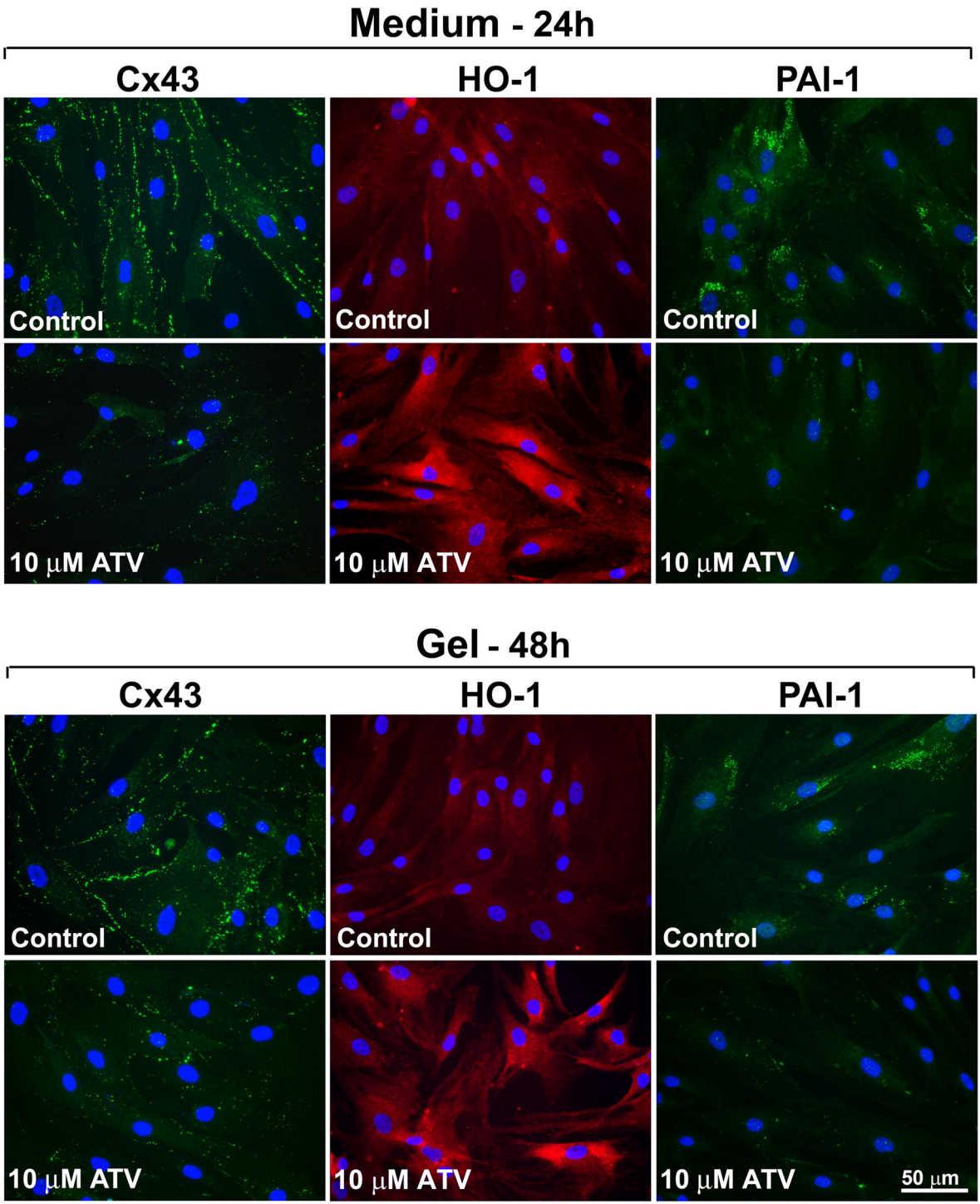

Control
5μM ATV
10μM ATV
Relative protein expression
(normalized to α-Tubulin)
HO-1
PAI-1
MMP9
Cx43

Figure 7


Medium - 24h
Cx43
HO-1
PAI-1
Control
Control
Control
10 μM ATV
10 μM ATV
10 μM ATV
Gel - 48h
Cx43
HO-1
PAI-1
Control
Control
Control
10 μM ATV
10 μM ATV
10 μM ATV
50 μm