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HMGB1 protein inhibitors and/or antagonists for the treatment of vascular diseases

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(54) Title: HMGB1 PROTEIN INHIBITORS AND/OR ANTAGONISTS FOR THE TREATMENT OF VASCULAR DISEASES

(57) Abstract: The use of HMG box-binding molecules and molecules having sequence homology with HMG box for the preparation of therapeutic agents for the treatment of vascular diseases is described.

HMGB1 PROTEIN INHIBITORS AND/OR ANTAGONISTS FOR THE
TREATMENT OF VASCULAR DISEASES.

The present invention concerns the field of
molecular biology and more particularly HMGB1 protein
inhibitors and HMGB1 antagonists to be used for the
treatment of vascular diseases, including those due to
5   angioplasty.

HMGB1 protein (known, before 2001, as HMG; Bustin,
2001, Trends Biochem. Sci., 26, 152-153) is the
archetypal protein of the HMG-box family, which is
characterised by the presence of DNA binding domains,
10 called HMG boxes. HMG1 is a small 25-kD protein, of 215
amino acids, with a highly conserved sequence among
mammals. The HMGB1 molecule is organised into three
domains: two DNA binding domains, HMG Box A and Box B,
which are followed by an acidic COOH terminus composed
15 of 30 glutamic and aspartic residues. The two HMG
boxes, box A and box B, are 80 amino acid segments (29%
identical, 65% similar), having an L-shaped
tridimensional structure (Hardman et al., 1995,
Biochemistry, 34:16596-16607; Read et al., 1993,
20 Nucleic Acids Res., 21:3427-3436; Weir et al., 1993,
EMBO J., 12:1311-1319).

HMGB1 has originally been identified as a ubiquitously
expressed, abundant nuclear protein. It is present in
more than 1 million copies per single nucleus and binds
25 double stranded DNA without sequence specificity.
Instead, HMGB1 binds with high affinity to specific DNA

structures like kinked or bent DNA and four-way
junctions. However, HMGB1 can be recruited to double
stranded DNA by interaction with several different DNA-
binding proteins. When bound to double stranded DNA, it

5   induces structure distortion, allowing the formation of
nucleoprotein complexes where several DNA-binding
proteins can contact each other while bound to their
respective DNA cognate sites (Müller et al., 2001, EMBO
J., 16: 4337-4340 and other reference cited

10 herewithin). The phenotype of Hmgb1 -/- mice is in
agreement with this model (Calogero et al., 1999, Nat.
Genet., 22:276-280).

Recently, an additional role for HMGB1 outside the cell
nucleus has come into focus: HMGB1 works as late

15 mediator of endotoxin-induced lethality as well as
acute lung inflammation in mice; as well the elevated
serum level of HMGB1 in septic patients is a prognosis
marker (international patent application No. WO
00/47104). HMGB1 can be secreted by macrophages and

20 pituicytes in culture in response to cytokines and
bacterial endotoxin (Abraham et al., 2000, J. Immunol.,
165: 2950-2954; Wang et al., 1999, Surgery (St. Luis),
126:389-392; Wang et al., 1999, Science, 285:248-251).
The release of HMGB1 from murine erythroleukemia cells

25 is correlated with cell differentiation and the protein
can be found in a plasma membrane-associated form in
these cells (Passalacqua et al., 1997, FEBS Lett.,
400:275-279; Sparatore et al., 1996, Biochem. J.,
320:253-256). A protein called amphoterin, identical in

30 sequence to HMGB1, has been described in the brain,

where it is found in the nucleus and cytoplasm of neuronal cells as well as in the extracellular space. If exogenously added, HMGB1 mediates outgrowth of neurites, and laminin-dependent migration of
5   neuroblastoma and glioma cells is inhibited by antibodies against HMGB1 (Fages et al., 2000, J. Cell Sci., 113:611-620; Merenmies et al., 1991, J. Biol. Chem., 266:16722-16729; Parkkinen et al., 1993, J. Biol. Chem., 268:19726:19738; Rauvala et al., 1988, J.
10   Cell Biol., 107:2293-2305). Interactions between HMGB1 and the plasminogen activation system, in particular t-PA (tissue-type plasminogen activator), results in enhanced plasmin formation (Parkkinen and Rauvala, 1991, J. Biol. Chem., 266: 16730-16735). Degradation of
15   extracellular matrix proteins is an important step in the cell migration process, and HMGB1-promoted increase of extracellular protease activity might enable the cells to migrate.

HMGB1 has been identified as one of the ligands
20   binding to the RAGE receptor (Receptor for advanced glycation endproducts) (Hori et al., 1995, J. Biol. Chem., 270: 25752-25761). RAGE is a multiligand receptor of the immunoglobulin superfamily and is expressed in many cell types, including endothelial
25   cells, smooth muscle cells, mononuclear phagocytes, and neurons (Brett et al., 1993, Am. J. Phathol., 143: 1699-1712; Neeper et al., 1992, J. Biol. Chem., 267: 14998-15004). It is implicated in several different pathological processes, such as diabetes, amyloidoses,
30   and atherosclerosis (Schmidt et al., 1999, Circ. Res.,

84: 489-497). Interaction of HMGB1 and RAGE induces neurite outgrowth, and the two proteins colocalize at the leading edge of advancing neurites during embryonic development (Huttunen et al., 1999, J. Biol. Chem.,
5   274:19919-19924). The block of tumour growth and metastasis is observed preventing the interactions between HMGB1 and RAGE; moreover, inhibition of this interaction suppresses activation of mitogen-activated protein (MAP) kinases and the expression of matrix
10   metalloproteinases, molecules importantly linked to tumour proliferation and invasion (Taguchi et al., 2000, Nature, 405: 354-360).

Moreover, it is already known that inhibitors of HMGB1 (i.e. Glicyrrhizin) can be used as anti-
15   inflammatory agents (Sakamoto et al., 2001, Biol. Pharm. Bull., 24(8) 906-911; Yoh et al., 2002, Dig. Dis. Sci., 47(8), 1775-1781).

The inventors of the present invention, demonstrated that HMGB1 has a potent biological effect
20   on smooth muscle cells (SMC), one of the cell types where RAGE is expressed on the surface. Vascular SMC cells are the most predominant cells of the larger blood vessels; they are located in the tunica media where are embedded in the extracellular matrix. In
25   intact vessels, SMC cells are in a contractile state and show a phenotype characterised by the absence of cell division and migration responsible for vessel wall rigidity and elasticity maintenance and blood pressure control.

When the endothelium is damaged, either after mechanical or inflammatory injuries, SMC cells switch to a synthetic phenotype and undergo cell division and cell migration. The migration of SMC cells from the tunica media to the tunica intima, resulting in intimal thickening, plays an important role in the

pathophysiology of many vascular disorders, such as atherosclerosis and restenosis after coronary angioplasty. In the synthetic state, SMC cells also produce higher amounts of extracellular proteinases,
5   growth factors, and cytokines and secrete a fibrous extracellular matrix. After vessel wall injury, the release of several growth factors and/or chemoattractants either by circulating monocytes, macrophages and platelets, or by damaged endothelial
10 cells can induce SMC cells switch from the contractile to the synthetic phenotypes and it can direct the migration of SMC cells towards the vessel intima. Among these factors, bFGF appears to be one of the most important, but however, SMC cells can also start
15 migration in response to angiogenic stimuli (Schwartz, 1997, J. Clin. Invest., 99:2814-2816; Van Leeuwen, 1996, Fibrinolysis, 10:59-74).
Trying to define the effect and the mechanism by which HMGB1 induces RSMC cell migration, the inventors
20 demonstrated that HMGB1 is a strong chemoattractant and it induces their cell shape changes, and cytoskeleton reorganisation. These events are inhibited by addition of an anti-RAGE antibody and by pertussis toxin, underlining that both RAGE and a Gi/o protein might be
25 involved in the pathway. Furthermore, the evidence that HMGB1 promotes the translocation of phosphorylated ERK 1 and 2 proteins into the nucleus, indicates the involvement of the MAP kinase pathway. Then, it has been demonstrated that HMGB1 is released by damage or

necrosis of a variety of cell types, including endothelial cells.

Therefore, HMGB1 has all the hallmarks of a molecule that can promote atherosclerosis and restenosis after

5   vascular damage.

The inventors also demonstrated that HMGB1 fragments, corresponding to HMG boxes, are more efficacious than the entire full-length molecule and even HMG box domains of other proteins of the HMG-box

10 family can induce the same effects.

Consequently, every kind of molecules able to block the interaction between HMGB1 and its RAGE receptor (i.e. all the molecules belonging to the inhibitors class: antibodies or antibodies fragments, fourway DNA;

15 and all the molecules belonging to the HMG box-antagonist class: HMGB1 fragments molecules containing the HMG box domain) can efficiently be used for the production of pharmacological preparation in order to avoid, retard or inhibit atherosclerosis and restenosis

20 after vascular epithelium damage even due to angioplasty.

HMGB1-binding molecules or HMGB1 inhibitors can be injected or released by instruments used for angioplastic surgery, or said molecules can be bound to

25 the instruments' surface.

Object of the present invention is the use of molecules able to block the interaction between HMGB1 and RAGE for the preparation of therapeutic agents for the treatment of vascular diseases.

Thus, in accordance with a first embodiment of the invention, there is provided a method of treating vascular diseases excluding the consequences of diabetes, said method comprising administering to a subject an HMG box-binding molecule.

In a second embodiment, the invention provides a method of treating vascular diseases excluding the consequences of diabetes, said method comprising administering to a subject an antagonist molecule having sequence homology with HMG box, not including sRAGE, and being able to bind the functional HMG box binding domain of RAGE receptor.

In a third embodiment, the invention provides a method of promoting connective tissues remodelling, said method comprising administering to a subject an HMGB1 and/or HMGB1 fragment corresponding to HMG box, an HMG box domain of any other protein belonging to HMG-box family, or any other protein of the HMG-box family.

20 In a fourth embodiment, the invention provides a method of treating wounds, burns or bedsores, or any combination thereof, said method comprising administering to a subject an HMG box-binding molecule.

In a fifth embodiment, the invention provides a method of reducing connective tissues remodelling, said method comprising 25 administering to a subject an antagonist molecule having sequence homology with HMG box and being able to bind the functional HMG box binding domain of RAGE receptor.

In a preferred embodiment of the invention said
molecules are released by catheters, surgical
instruments or stents for angioplasty, during or after
said operation.

5 Further features and advantages of the invention will
be more readily apparent from the following detailed
description with reference to the accompanying
drawings.

In the drawings:

10   Figure 1 shows HMGB1 chemotactic activity on RSMC
in chemotaxis assays performed using modified Boyden
chambers. The value of 100% corresponds to the number
of cells migrating in the absence of any stimulator
(random cell migration). The data represent the mean $\pm$

15 SD (n=3). Figure 1-A shows concentration-dependent
migratory response of RSMC to HMGB1 purified from calf
thymus. Figure 1-B shows the comparison of the
chemotactic effect of HMGB1 proteins, either purified
from calf thymus or expressed in yeast, with those of

20 chemoattractants fMLP and bFGF. Figure 1-C shows the
effect of anti-HMGB1 antibodies on fMLP and HMGB1-
induced migration. The asterisk (*) indicates
treatments where the migratory response was
statistically different from the control beyond the

25 p=0.0001 limit in Student's test. Figure 1-D shows the
concentration-dependent migratory response of RSMC to
HMGB1 expressed in yeast (Pichia pastoris).

Figure 2 shows the effect of HMGB1 on RSMC
morphology and cytoskeleton organization. Figure 2-A

30 shows the effect of HMGB1 purified from calf thymus or

expressed in yeast or in E. coli on subconfluent
cultures of RSMC. Actin filaments were visualised using
TRIC-phalloidin. Figure 2-B shows how anti-HMGB1 rabbit
antibodies inhibit HMGB1-stimulated cytoskeleton
5 reorganization. Resting cells (state 1) exhibit
numerous stress fibers. Nonresting cells (state 2) show
a reorganization of actin cytoskeleton.

Figure 3 shows the chemotactic response of RSMC to
the HMG box domains of HMGB1. Figure 3-A shows the
10 concentration-dependent response to Box A e Box B, both
expressed in E. coli. Random cell migration is referred
to as 100% migration. The data represent the mean $\pm$ SD
(n=3). The statistical significance of the result is
p<0.0001 in a ANOVA model, for both Box A and Box B.
15 Figure 3-B shows the effects of full-length HMGB1
expressed in E. coli, Box A+B, Box A or Box B on actin
cytoskeleton organization. Actin filaments were
visualized using TRIC-phalloidin.

Figure 4 shows the effects of HMGB1 and its HMG
20 boxes on RSMC migration into a wound. The value of 100%
corresponds to the number of cells migrating in the
absence of any stimulator (basal migration). The data
represent the mean $\pm$ SD (n=5). Statistical significance
is 0.05<p<0.01 for the treatment with bFGF and full-
25 length bacteria-made HMGB1, 0.01<p<0.001 for the
treatment with Box A and Box B and 0.001<p<0.0001 for
the treatment with calf thymus HMGB1.

Figure 5 shows how HMGB1 binds to the surface of
RSMC and stimulates cell motility through RAGE. Figure
30 5-A shows that large amounts of HMGB1 bind to the

surface of RMSC. In figure 5-B RSMC expressing RAGE are shown. Figure 5-C shows how anti-RAGE antibody inhibits HMGB1-induced RSMC migration. Statistical significance is $0.001 < p < 0.0001$ for treatment with HMGB1 and HMGB1

5   plus unspecific antibody.

Figure 6 shows how pertussis toxin (PT) inhibits HMGB1-induced RSMC migration and actin cytoskeleton reorganization. In figure 6-A chemiotaxis assays performed using modified Boyden chambers are shown. The

10   value of 100% corresponds to basal cell migration in the absence of any stimulator; the data represent the mean $\pm$ SD. Figure 6-B shows evident cytoskeleton reorganization, actin filaments were visualised using conjugated TRITC-phalloidin.

15   Figure 7 demonstrates that the MAP kinase pathway is involved in HMGB1 signaling. Cells are stained with specific antibody against phosphorylated ERK1/2 and DAPI, and a separate sample of cells is stained with TRITC-phalloidin to visualize the reorganization of the

20   cytoskeleton.

Figure 8 shows that HMGB1 is released by necrotic and damaged cells. Figure 8-A shows the results of Western-blot analysis of proteins released by necrotic or permeabilized HeLa; HMGB1 presence is evident in

25   line 1 and line 3. Figure 8-B shows the results of immunofluorescence assays performed on necrotic and living HeLa.

Figure 9 shows that HMGB1 is present in the nuclei of endothelial cells, but not in those of vascular SMC.

30   In figure 9-A and in figure 9-B it is shown that HMGB1

is present in the nuclei of endothelial cells but it is
not detectable in the nuclei of vascular smooth muscle
cell of a section of human pancreatic artery stained
with anti-HMGB1 antibody and counterstained with
5   ematoxylin, at low (A) and high (B) magnification. The
red frames indicate the location of the area shown in
figure B and the arrows point to the nuclei of SMC. In
figure 9-C Western blot analysis shows expression level
of HMGB1 in RSMC in comparison to HeLa cells.

10   Figure 10 shows the chemotactic effect of HMGB1 on
mouse embryonic fibroblasts in chemotaxis assays
performed using modified Boyden chambers, in the
presence or in the absence of anti-RAGE antibodies
(1000 ng/ml). The value of 100% corresponds to the
15   number of cells migrating in the absence of any
stimulator (random cell migration). The data represent
the mean ± SD (n=3).

Expression and purification of HMGB1 and derivates
20   In the first step, it has been necessary to express
and purify HMGB1 and derivates.

         Expression of full-length HMGB1 was performed in E.
Coli transformed with pT7-7-rHMG1cm plasmid (kind gift
of Prof. J.O. Thomas, Cambridge University) and
25   purification was performed following a well-known
protocol (Müller et al., 2001, Biochemistry, 40: 10254-
10261).

         Expression and purification of full-length HMGB1 in
yeast Pichia Pastoris were performed following a well-

known protocol (Mistry et al., 1997, Biotechniques, 22:718-729).

The well-known plasmids pRNHMG1/M1-V176, pT7HMG1bA and pT7HMG1bB were used for the expression and
5 purification of BoxA + BoxB, BoxA and BoxB respectively following well-known procedures of purification of the single and double boxes (Bianchi et al., 1992, EMBO J., 11: 1055-1063).

To demonstrate chemotactic effect of HMGB1, three
10 independent cell migration assays were performed: chemotaxis assay, chemokinesis assay and in vitro wounding assay. The functional relationship between HMGB1-induced cell migration and morphological changes (i.e. actin fibers reorganization, cell elongation and
15 cell's shape polarization) of nonresting cells was investigated.

Chemotaxis assay

Chemotaxis assays were performed using well-known
20 protocols (Degryse et al., 1999, Blood, 94:649-662). Modified Boyden chambers were used with filters having 0.5 μm pore size (Corning, Acton, MA) and treated with collagen I (100 μg/ml in 0.5 M acetic acid) e fibronectin (10 μg/ml) (Roche). RSMC cells (kind gift
25 of Dr. Marco Bertulli, Bayer Research Laboratories, Milan) were cultured in DMEM serum-free and a sample of 20.000-40.000 cells was added to the upper well of Boyden chamber. The molecules to be tested were diluted in the same serum-free medium and added to the lower
30 well.

Different HMGB1 preparations were used: HMGB1
purified from calf thymus (kind gift of J. Bernués,
C.S.I.C., Barcelona, Spain), E. coli expressed
recombinant-HMGB1, and a lightly modified HMGB1 form
5   (containing EAEAYVEF aminoacids bound to the N-
terminus) produced in yeast Pichia pastoris (Mistry et
al., 1997, Biotechniques, 22:718-729).

If necessary, the polyclonal rabbit anti-HMGB1
(Pharmingen BD, Torrey Pines, CA), the pertussis toxin
10   (PT) from Bordetella pertussis (kind gift of Dr. M.G.
Pizza, I.R.I.S., Siena) or the inhibitors were added in
both wells.

Overnight cell migration was allowed at 37°C, then
cells remaining on the upper surface of filters were
15   scraped off and filters were fixed in methanol and
stained in a solution of 10% crystal violet in 20%
methanol. All experiments were performed at least twice
in triplicate.

Results, as shown in figure 1-A, 1-B, 1-C, 1-D, are the
20   mean ± SD of the number of cells counted in 10 high
power fields per filter and expressed as fold over
control. To random cell migration (i.e., migration in
the absence of chemoattractant) was given the arbitrary
value of 100%.

25   Statistical analysis was performed using Student's t
test for pairwise comparisons of treatments, or an
ANOVA model for the evaluation of treatments with
increasing doses of a reagent.

HMGB1 from calf thymus stimulates migration of RSMC
30   in a concentration-dependent manner, starting at doses

as low as 0.1 ng/ml and with a 2.5-fold maximal
response at 100 ng/ml (Figure 1-A). The effect of HMGB1
is comparable in amplitude to the effects of the well-
characterised attractants fMLP and bFGF (Figure 1-B).
5   Polyclonal antibodies against HMGB1, but not
nonspecific control antibodies, totally block the
migratory response (Figure 1C), showing that this is
specifically due to HMGB1. These antibodies fail to
alter the effect of the chemoattractant peptide fMLP
10 used as positive control. Similar results are obtained
with recombinant HMGB1 produced in yeast P. pastoris
(Figure 1-D).

Immunofluorescence assay
15   Samples of 15.000-20.000 RSMC, 20-40% confluence,
were seeded on glass coverslips in 2 cm^2 wells and
cultured for 24 hours in DMEM plus 10% FCS, washed with
PBS, and cultured for another 24 hours in DMEM without
FCS. RSMC were stimulated with HMGB1 100 ng/ml for
20 increasing time intervals, from 5 to 120 minutes at
37°C. After stimulation, RSMC were fixed for 20 minutes
at room temperature with a solution of 3%
paraformaldehyde, 2% sucrose in PBS, pH 7.5, followed
by three washes with PBS-BSA 0.2%. Cells were
25 permeabilized with 20 mM Hepes pH 7.4, 300 mM
saccharose, 50 mM NaCl, 3 mM MgCl$_2$, 0.5% (v/v) Triton
X-100 for 3 minutes at 4°C, and washed again three
times with PBS-BSA 0.2%. Then, RSMC were incubated with
PBS-BSA 2% for 15 minutes at 37°C, with primary
30 antibodies for 30 minutes at 37°C, washed three times

with PBS-BSA 0.2%, and further incubated with PBS-BSA
2% for 15 minutes. At the end, cells were stained with
secondary antibodies and/or phalloidin conjugated with
rodamin for visualization of filamentous actin; in some
5   cases, DAPI (4',6-diamidino-2-phenilindolo, Roche) was
used to label the nucleus.

After all the subsequent incubation, coverslips were
washed three times with PBS-BSA 0.2%, twice with
distilled water, mounted with 20% Mowiol in PBS and
10   analysed on a Axiophot microscope (Carl Zeiss).
Fluorescence photographs were taken either on T-Max 400
or EPH P1600X film (Eastman Kodak) using Zeiss 40 and
100 neofluar lenses.

Low magnification pictures, in figure 2-A, show
15   that stress fibers content, cell shape and size, and
cytoskeleton organization change within 30 minutes, but
reverse after 120 minutes. Higher magnification
pictures (figure 2-B) show that before stimulation are
well-visible numerous stress fibers and the cell shape
20   is a nonpolarized. Within 15-30 minutes, a complete
change of morphology and cytoskeleton organization
occurs: RSMC show an elongated, polarized morphology
that reflected the spatial rearrangement of the actin
cytoskeleton. The effects of HMGB1 slowly decrease:
25   After 1-2 hours, the stress fiber content increases
back to the initial level and cell morphology returns
similar to that of unstimulated control cells.

In certain experiments cells were overnight
pretreated with antibodies or PT or inhibitors. As
30   shown in figure 2-B, antibodies against HMGB1 totally

inhibit the cytoskeletal reorganization and the
morphological change of RSMC induced by HMGB1. Control
antibodies are not able to inhibit HMG1 effects.

Finally, to determine whether the observed effects
5   of HMGB1 on RSMC actually reflect a dynamic transition
from resting to motile states, the proportion of cells
in each different state was quantified. Low
magnification pictures were taken and the cells were
classified in two states:

10   - state 1, where cells show the appearance typical
of unstimulated cells characterized by a high number of
stress fibers and a nonpolarized cell shape;

- state 2, where RSMC exhibiting a low stress
fibers content, membrane ruffling, actin semi-rings, or
15 an elongated shape.

It is clearly shown in figure 2-C that in
unstimulated cultures 60% of the cells are in state 1
and 40% in state 2; within 5 minutes after stimulation,
the proportion of cells in state 2 increases to 60%,
20 and rose to 80% after 15-30 minutes. One hour after
stimulation with HMGB1, these proportions reverse back
to the values of unstimulated cultures, with 60% of
RSMC in state 1 and 40% in state 2. These data
demonstrate that HMGB1 effects are transient and
25 represent the change from a resting to a migrating
state, these data confirm chemotaxis results: HMGB1 is
a chemoattractant for RSMC.

In vitro wounding assay

Confluent cultures of RSMC, grown on glass coverslips
in 2-cm^2 wells, were washed once with PBS and FCS-
starved for 24 hours in serum-free DMEM. Then to
simulate the wound, a single line was made with the tip
5   of a pipette in the central region of the monolayers.
The so treated monolayers, were washed once with PBS
and are allowed to recover for 48 hours in serum-free
medium supplemented or not with HMGB1 (100ng/ml). Then
cells were fixed and stained with TRITC-phalloidin.
10 Quantification of the migration was made by taking
photographs at lower magnification and by counting the
number of cells that had migrated into the cell-free
space. The data represent the mean $\pm$ SD and the value
100% corresponds to the number of cells migrating in
15 the absence of any stimulator (basal migration).

As shown in figure 4, HMGB1 stimulation increases
the number of migrating cells by 5-2-fold. Box A and
Box B (10 ng/ml) were also tested and both stimulate
cell migration 1.8-fold. Finally, the comparison with
20 bFGF (50 ng/ml) underlines that the above mentioned
molecules are more effective. It is possible to assume
that wound healing is based on the same signaling
pathway of chemotaxis and chemokynesis.

25 Signaling pathway

Afterwards, signaling pathway has been detected.

To act as a migratory signal, HMGB1 must arrive to
the membrane of responsive cells and bind to a
receptor. To test whether HMGB1 binds to the surface of
30 RSMC, one million cells were trypsinized and incubated

for 20 minutes at 4°C in PBS containing 800 ng of the
Box A+B peptide and 5 µg BSA. The BoxA+BoxB polypeptide
is slightly smaller than the endogenous full-length
HMGB1 and can thus be distinguished easily on SDS-PAGE
5   gels. Then, cells were pelleted and the supernatant was
saved; after two washes in 500 µl cold PBS, cells were
resuspended in SDS-PAGE sample buffer, heated for 5
minutes at 100°C and loaded on 12% tricine-SDS gel
(line P), adjacent to 20 µl of supernatant (line S).
10   Then, the gel was blotted to a Immobilon filter, which
was stained with India ink.

     In figure 5-A a SDS-PAGE gel is shown, from which
the amount of Box A+B recovered in the cell pellet and
in the supernatant can be calculated, and it can be
15   estimated that more than 500 000 Box A+B molecules bind
to a single RSMC. This result demonstrates that
extracellular HMGB1 can bind to RSMC, but most likely
does not reflect the actual receptor number. Indeed,
HMGB1 has already been shown to bind to heparin and
20   proteoglycans (Bianchi, 1988, EMBO J., 7: 843-849; Nair
and Jungalwala, 1997, J. Neurochem., 68: 1286-1297;
Salmivirta et al., 1992, Exp. Cell Res., 200: 444-451);
thus, HMGB1 might also be associated with the
extracellular matrix produced by RSMC, as already
25   demonstrated by the inventors in HeLa, where only small
amounts of HMGB1 bind to cells because these cells
produce little extracellular matrix.

     HMGB1 has been reported to bind to RAGE that is
expressed by a vast range of cell types. To demonstrate
30   that RAGE is present on RSMC membrane, one million RSMC

were lysed in a plate containing SDS-PAGE sample buffer
(50 mM Tris pH 6.8, 2% 2-mercaptoethanol, 4% SDS, 12%
glicerol, 0.05% bromophenol blue), denaturated for 5
minutes at 100°C and separated on 12 % acrylamide.

5   Separated   proteins   were   blotted   on   Immobilon
(Millipore) membrane using a tankblot system 25 mM Tris
pH 7.5, 0.192 M glycine, 20% methanol. The blot was
blocked for one hour at room temperature in 5% skim
milk/TBST (20 mM Tris, pH 7.5, 137 mM NaCl, 0.1% Tween

10   20), three time washed in TBST, and incubated with
anti-HMGB1 antibody in TBST-0.01% BSA. Incubation with
secondary antibody was conducted after washing with
TBST-0.01% BSA. Proteins were detected with ECL system
(Amersham). The presence of RAGE was detected using

15   anti-RAGE antibody (kind gift of Dr. A.M. Schmidt,
Columbia University, NY). Results shown in figure 5-B
demonstrate that RAGE is present on RSMC. Furthermore,
HMGB1-induced chemotaxis is not only inhibited by anti-
HMGB1 antibodies but also by anti-RAGE antibodies, as

20   shown in figure 5-C. Anti-RAGE antibodies block
cytoskeletal reorganization and morphological changes
of RSMC in response to HMGB1 migratory signal;
irrelevant antibodies are not able to block
cytoskeleton reorganization.

25   These data indicate that the RAGE receptor is
required for the HMGB1-induced responses of RSMC.

        Knowing that many chemoattractants act via membrane
receptors associated to heterotrimeric GTP binding
proteins (G proteins), whether G proteins could be

30   implicated in HMGB1 signaling was tested. The pertussis

toxin (PT) was used because it inhibits a specific
subclass of G proteins, the Gi/o proteins, and it
reveals their involvement in the signaling pathway
(Baggiolini et al., 1994, Adv. Immunol., 55:97-179;
5   Haribabu et al., 1999, J. Biol. Chem., 274:37087-
37092). mPT, an inactive mutant of PT, was used as a
control. RSMC were pre-treated with PT or with mPT (50
ng/ml) for 6 hours, thus stimulated with HMGB1 (100
ng/ml), BoxA or BoxB (10 ng/ml) for 30 minutes.
10 Chemotaxis assays were performed as previously
described. The data represent the mean $\pm$ SD and the
value of 100% corresponds to basal migration in the
absence of any stimulator. In figure 6-A the inhibitory
effect of PT on HMGB1-induced chemotaxis is shown.
15 These data suggest the involvement of $G_{i/o}$ proteins in
the signaling pathway controlled by HMGB1. In figure 6-
B the cytoskeleton reorganization is shown, actin
filaments were visualised as previously described.
Afterwards, whether the HMGB1-induced signaling
20 involves the MAP kinase pathway, was investigated; in
fact, it is known that these proteins are activated by
RAGE, and they have a direct role in the regulation of
the intracellular motility machinery. RSMC were pre-
treated with PD98059 (50 mM) for one hour or were not
25 pre-treated, stimulated for 30 minutes with HMGB1 from
calf thymus (100 ng/ml) and stained with specific
antibodies against phosphorylated ERK1/2 (New England
Biolabs, Beverly, MA) and DAPI. A separate sample of
cells was stained with TRITC-phalloidin to visualize
30 the reorganization of cytoskeleton. In figure 7 is

shown how, within 30 minutes, HMGB1 stimulation induces
the activation of ERK1/2 proteins in RSMC and induces
their   nuclear   translocation;   in   contrast,
phosphorylated ERK proteins are hardly detectable and
5   located   in   the   cytoplasm,   in   unstimulated   RSMC.
Moreover, PD98059, the selective inhibitor of MEK, the
upstream regulator of ERK, inhibits HMGB1-induced ERK
phosphorylation and nuclear translocation, as well as
RSMC   migration   and   citoskeleton   reorganization.
10   Consequently, these data show that the MAP kinase
pathway plays an essential role in HMGB1-induced cell
migration.

Induction of cell damage
15   Considering the state of the art, has been detected
whether damaged cells or cells undergoing necrosis
could release HMGB1 in the extracellular medium.
         HeLa cells and HUVEC were induced to undergo
necrosis by treatment with 5 μM ionomycin (Sigma) and
20   20 μM CCCP, or mM deoxyglucose and 10 mM sodium azide.
After 16 hours at a 37°C, the number of cells
undergoing necrosis was scored morphologically, and
when it approached 50% the supernatant was collected.
         For Western blot analysis, the medium from treated
25   and untreated cells was collected and concentrated 50-
fold using Amicon Ultrafree-MC filters; the cells were
dissolved in the SDS-PAGE sample buffer.
         For immunofluorescence analysis, the cells were
fixed with 4% PFA, incubated with an anti-HMGB1
30   antibody, and stained with secondary antibody and DAPI.

The permeabilization of cells was performed with using 0.1% NP-40 in PBS.

In figure 8-A, Western-blot analysis of protein in supernatants (S) and cell pellets (P) is represented, HMGB1 was recovered in the supernatant of both necrotic cells and damaged cells. In figure 8-B immunofluorescence assays performed on single living and necrotic HeLa is shown, HMGB1 is not associated to the remnants of necrotic cells.

In figure 9 the results of immunohistochemistry assays are shown, these data confirm that HMGB1 is contained in the nuclei of endothelial cells that line human arteries but not in the nuclei of RSMC (figure 9-A low magnification; figure 9-B high magnification), in fact, most nuclei of smooth muscle cells contain undetectable amounts of HMGB1 (frame in Figure 9-B). In figure 9-C, Western-blot analysis shows the expression level of HMGB1 in RSMC in comparison to HeLa cells, and it demonstrates that in vitro cultures of RSMC contain low amounts of HMGB1 in comparison to HeLa cells.

Altogether, these data suggest that the HMGB1 molecules that signal to vascular smooth muscle cells may originate simply by necrosis or mechanical damages of nearby cells.

In conclusion, the above mentioned experimental data, foundations of the present invention, demonstrate that nuclear HMGB1 protein is a strong mediator of vascular remodeling occurring after mechanical damage and/or inflammation and can be passively released by damaged or necrotic cells.

In particular these data suggest what follows:

HMGB1 ACT AS A CHEMOATTRACTANT

HMGB1 is a potent chemoattractant as bFGF or fMLP
in chemotaxis assays and wounding assays, and promotes
changes of cell shape and of cytoskeleton organization
similar to those observed with pro-urokinase; these
effects are specifically due to HMGB1 and not to
potential contaminants. In addition, antibodies
directed against HMGB1 inhibit its effects on cell
migration, whereas nonspecific control antibodies are
unable to do so.

BINDING TO RAGE INITIATES THE HMGB1 SIGNALING PATWAY IN
RSMC

The above reported experiments show that RAGE is
expressed in RSMC, and anti-RAGE antibodies inhibit the
effect of HMGB1 on RSMC.

It was confirmed that MAP kinases are involved in
HMGB1-induced cell migration of RSMC, since ERK1/2 are
phosphorylated and translocated to the cell nucleus
upon HMGB1 stimulation, and the MEK inhibitor PD98059
is able to block HMGB1-induced cell migration. Data
also indicate that a $G_{i/o}$ protein is involved in the
process which is activated by HMGB1, since HMGB1-
induced cell migration can be blocked by Bordetella
pertussis toxin. G protein are usually associated to
seven-trasmembrane-elix receptors (7TM), but so far no
direct association between RAGE and G protein has been
described. Up to now, it is unknown if HMGB1 need to

bind a 7TM receptor/G protein receptor in addition to RAGE, or if a G protein is involved downstream to RAGE, or in a feedback mechanism.

5   HMGB1 PARACRINE FUNCTION

HMGB1 is released in a unregulated manner, which means upon stimulation with cytokines or lipopolysaccharide, when cells are mechanically damaged or undergo necrosis. Thus, HMGB1 can signal the damage or

10   destruction of an individual cell to the neighbouring cell in a paracrine manner. The cells that respond to extracellular HMGB1 appear to contain very little HMGB1 themselves, and almost none in the nucleus. RSMC contain very little HMGB1 compared with HeLa cells or

15   endothelial cells, and what little HMGB1 they contain is mainly located in the cytoplasm. Migrating RSMC tend to concentrate HMGB1 on their surface at the leading edge of the cell. It can be supposed that HMGB1-responsive cells could contain little HMGB1 to reduce

20   the chance of inappropriate responses to their own HMGB1. Concentration of HMGB1 at the leading edge of migrating cells might evoke HMGB1-induced responses in neighbouring cells: relocation of molecules involved in cell migration, such as integrins, the urokinase

25   receptor, or c-Src, is a feature of motile RSMC. Migration also involves the activation of extracellular proteases, and the interaction between HMGB1 and the plasminogen activation system might facilitate cell migration within the extracellular matrix.

30

ROLE OF HMGB1 IN VASCULOPATHIES

The responsiveness of smooth muscle cells to HMGB1,
the observation that endothelial cells contain high
amounts of HMGB1 while vascular SMC contain little, and
5   the release of HMGB1 from cells undergoing mechanical
damage, all the above results point to a possible role
of HMGB1 during the tissue remodeling occurring in
atherosclerosis and restenosis.

The above specified experimental results permitted
10   to identify the molecules, object of the invention,
able to inhibit the interaction between HMGB1 and RAGE
receptor; these molecules are classified, considering
their structural and functional characteristics, as
follows:

15   1. HMGB1 antagonists: HMGB1 fragments, HMG box
analogues, which can be more effective than the entire
full-length molecule, and proteins containing HMG box
domains, the last two are both able to bind to RAGE
receptor.

20   2. HMGB1 inhibitors: molecules, as antibodies or
antibody fragments and four-way DNA, which bind to HMG
box domain and avoid HMGB1 binding to RAGE.

These molecules are advantageously used for
pharmacological preparation which prevent, retard or
25   minimise atherosclerosis and/or restenosis after
vascular epithelium damages, including those events
that occur after angioplasty.

Furthermore, the inventors of the present invention
demonstrated that HMGB1 has a strong biological effect
30   on mouse embryonic fibroblasts. It is well known that

fibroblasts are the main cellular components of connective tissues and they are responsible for the synthesis and upkeeping of the connective extracellular matrix. More particularly, HMGB1 acts in vitro as a

5 potent chemoattractant for fibroblasts and anti-RAGE antibodies block said effect.

Consequently, every kind of molecules having homology with HMGB1 can be used, as the entire full-length protein, for the preparation of pharmacological

10 agents which positively regulate, thus facilitate and/or induce cellular migration of fibroblasts. In the same way, every kind of molecules able to block the interaction between HMGB1 and its RAGE receptor (i.e. all the molecules belonging to the inhibitors group:

15 antibodies or antibodies fragments, four-way DNA; and all the molecules belonging to the HMG box-antagonists group: HMGB1 fragments, molecules containing the HMG box domain) can efficiently be used for the production of pharmacological agents in order to avoid, retard or

20 reduce connective tissues regeneration.

An additional aim of the present invention is the use of HMGB1, HMGB1 fragments corresponding to HMG box, HMG box domains of other proteins belonging to the HMG-box family and other proteins of the HMG-box family,

25 for the preparation of therapeutic agents which facilitate and/or induce fibroblasts migration and consequently positively regulate connective tissues regeneration.

Its is an integral part of the present invention

30 the use of all the molecules, antagonists and/or

inhibitors, which inhibit the interaction between HMGB1 and RAGE receptor, for the preparation of therapeutic agents which reduce, retard, and avoid connective tissues regeneration, as focused by the following
5 experiments.

Chemotaxis assay on fibroblasts

Chemotaxis assays were performed using well-known protocols (Degryse et al., 1999, Blood, 94:649-662).
10 Modified Boyden chambers were used with filters having 0.5 μm pore size (Corning, Acton, MA) and treated with collagen I (100 μg/ml in 0.5 M acetic acid) and fibronectin (10 μg/ml) (Roche). Mouse embryonic fibroblasts were cultured following well-known
15 protocols (Calogero et al., 1999, Nat. Genet., 22:276-280) and after 24 hours of serum starvation, a sample of 20.000-40.000 cells was added to the upper well of Boyden chamber. E. Coli expressed recombinant-HMGB1 was diluted in the same serum-free medium and added to the
20 lower well.

Anti-RAGE antibodies (1000 ng/ml) (kind gift of Dr. A.M. Schimdt, Columbia University, NY) were added in both wells.

Overnight cell migration was allowed at 37°C, then
25 cells remaining on the upper surface of filters were scraped off and filters were fixed in methanol and stained in a solution of 10% crystal violet in 20% methanol. All experiments were performed at least twice in triplicate.

Results, as shown in figure 10 are the mean ± SD of
the number of cells counted in 10 high power fields per
filter and expressed as fold over untreated control. To
random cell migration (i.e. migration in absence of
5   chemoattractant) was given the arbitrary value of 100%.
Statistical analysis was performed using an ANOVA model
for the evaluation of treatments with increasing doses
of a reagent.

E. Coli expressed recombinant-HMGB1 stimulates
10 fibroblasts migration in a concentration-dependent
manner, starting at doses as low as 0,1 ng/ml and with
a maximal response at 100 ng/ml, at higher doses (1000
ng/ml) the response is lower than the control. Anti-
RAGE antibodies (1000 ng/ml) totally block the
15 migratory response (right side of the graphic of figure
10) showing that this is specifically due to HMGB1.

ROLE OF HMGB1 IN THE REGULATION OF CONNECTIVE
TISSUE REGENERATION.
20 The responsiveness of fibroblasts to HMGB1 points
out to a possible role of HMGB1 during connective
tissues remodelling occurring after damages due to
traumatic events or surgery. Moreover, the fact that
anti-RAGE antibodies block said response demonstrates
25 that the interaction between HMGB1 and RAGE receptor on
cellular surface is the basic event leading to
fibroblast sensitiveness to HMGB1.
In conclusion:
- HMGB1 and/or HMGB1 fragments corresponding to HMG
30 box, HMG box domains of other proteins belonging to

HMG-box family and other proteins of the HMG-box family are advantageously used for pharmacological preparations which positively regulate, i.e. facilitate and/or induce connective tissues regeneration.

5   - every kind of molecules able to inhibit the interaction between HMGB1 and RAGE, belonging to the antagonists group, (able to bond to RAGE receptor), and belonging to the inhibitors group, (i.e. able to bound the HMG box domain blocking HMGB1 bounding to RAGE

10   receptor) are advantageously used for pharmacological preparations which negatively regulate, i.e. block, retard or reduce connective tissues regeneration.

The claims defining the invention are as follows:

1. A method of treating vascular diseases excluding the consequences of diabetes, said method comprising administering to a subject an HMG box-binding molecule.

5   2. The method according to claim 1, wherein said molecule is selected from an antibody, an antibody fragment, an inhibitor or four-way DNA, but is not sRAGE.

3. A method of treating vascular diseases excluding the consequences of diabetes, said method comprising administering
10   to a subject an antagonist molecule having sequence homology with HMG box, not including sRAGE, and being able to bind the functional HMG box binding domain of RAGE receptor.

4. The method according to any one of the preceding claims, wherein said vascular disease comprises atherosclerosis and/or
15   restenosis that occurs during angioplasty.

5. The method according to any one of the preceding claims, wherein said molecule is released by a catheter, a surgical instrument or a stent for angioplasty.

6. A method of promoting connective tissues remodelling, said
20   method comprising administering to a subject an HMGB1 and/or HMGB1 fragment corresponding to HMG box, an HMG box domain of any other protein belonging to HMG-box family, or any other protein of the HMG-box family.

7. A method of treating wounds, burns or bedsores, or any
25   combination thereof, said method comprising administering to a subject an HMG box-binding molecule.

8. The method according to claim 7, wherein said molecule is selected from an antibody, an antibody fragment, an inhibitor or four-way DNA.

9.   A method of reducing connective tissues remodelling, said method comprising administering to a subject an antagonist molecule having sequence homology with HMG box and being able to bind the functional HMG box binding domain of RAGE receptor.
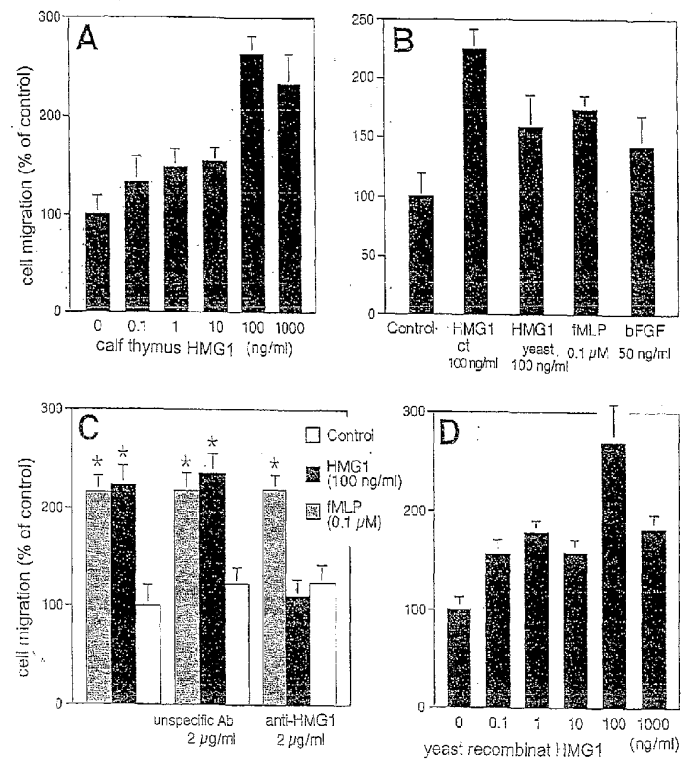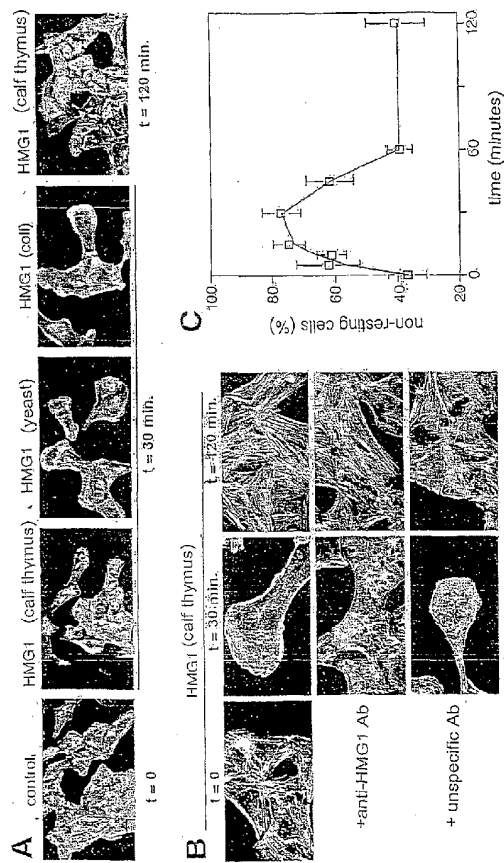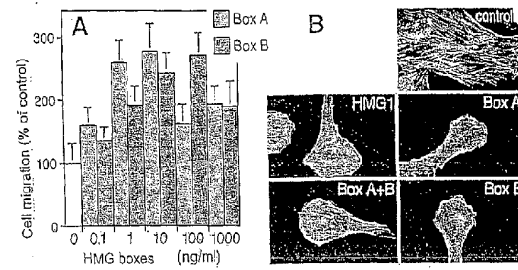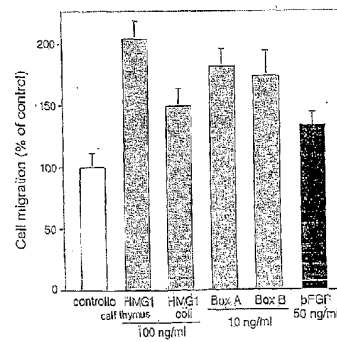
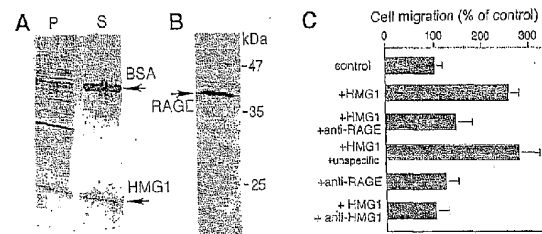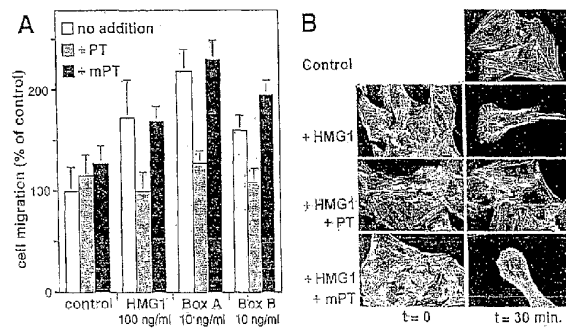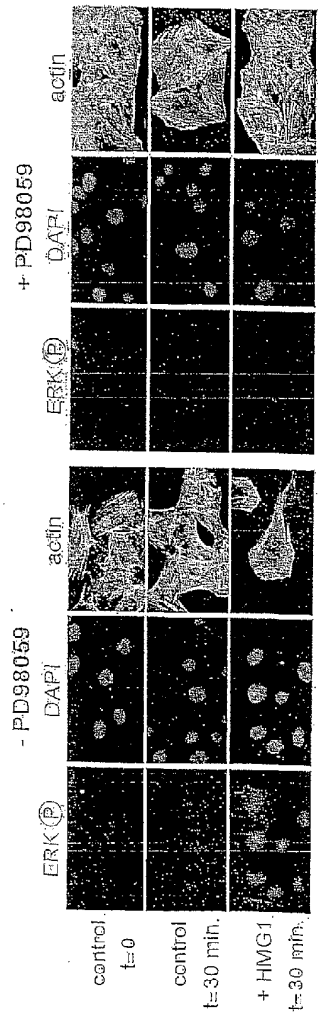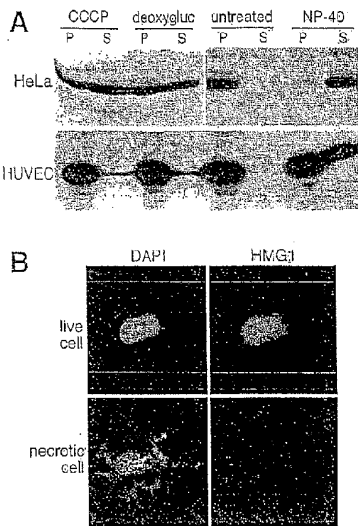5   Dated: 27 February 2008

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10