Study of the chromatin binding properties of the high mobility group 1 protein (HMGB1) in living and dead cells

Thesis

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STUDY OF THE CHROMATIN BINDING PROPERTIES OF THE HIGH MOBILITY GROUP 1 PROTEIN (HMGB1) IN LIVING AND DEAD CELLS

Thesis submitted in partial fulfilment of the requirement of the Open University for the degree of Doctor of Philosophy in Molecular and Cellular Biology

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ABSTRACT

HMGB1 (High mobility group 1 protein) is both a nuclear factor and a secreted protein. In the cell nucleus it acts as an architectural chromatin-binding factor that bends DNA and promotes protein assembly on specific DNA targets. Outside the cell, it binds with high affinity to RAGE (receptor for advanced glycation endproducts), and is a potent mediator of inflammation.

I used a GFP-fused HMGB1 in combination with photobleaching techniques to characterize the binding of the protein to chromatin. I found that the protein has differential chromatin binding properties in living, apoptotic, and necrotic cells. In living cells, HMGB1 exchanges rapidly between the soluble and the chromatin-bound states. In necrotic cells, HMGB1 is not associated with chromatin and is leaked into the extracellular medium. In apoptotic cells, HMGB1 is retained in the nucleus because it binds stably to chromatin, in a process that can be inhibited by trichostatin A and appears to be a consequence of histone H4 deacetylation.

The differential binding of HMGB1 to chromatin in necrotic and apoptotic cells is relevant with reference to the role of HMGB1 in promoting inflammation: as necrosis is always accompanied by inflammation in contrast to apoptosis, we made the hypothesis that HMGB1, released by necrotic cells but retained by apoptotic ones, could be used by proinflammatory cells as a signal to distinguish necrosis from apoptosis. Indeed, necrotic cells lacking HMGB1 are rather ineffective in activating proinflammatory cells, and injections of anti-HMGB1 antibodies in wt mice reduce inflammatory responses induced by liver necrosis, indicating that HMGB1 is necessary to promote inflammatory responses to necrosis. Moreover, if HMGB1 is detached from apoptotic chromatin by TSA treatment, also apoptotic cells become proinflammatory.

Thus, HMGB1 acts as a signal of unprogrammed cell death, and the retention of HMGB1 by apoptotic cells appears to be a safeguard mechanism to prevent unwanted inflammatory reactions in response to programmed cell death.

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LIST OF ABBREVIATIONS

AAP: acetaminophen

ALT: alanine aminotransferase

ACF: ATP dependent chromatin assembly and remodelling factor

BBP: bromophenol blue

BSA: bovine serum albumin

CAD: caspase-activated DNase

CCCP: carbonyl cyanide chlorophenylhydrazone

DAPI: 4',6-diamidino-2-phenylindole

DCs: dendritic cells

DMEM: Dulbecco's modified Eagle's medium

DTE: dithioerythritol

DTT: dithiothreitol

ELISA: enzyme linked immunosorbent assay

ER: endoplasmic reticulum

ERK: extracellular-signal Regulated Kinase

FBS: foetal bovine serum

FITC: fluorescein isothiocyanate

FLIP: fluorescence loss in photobleaching

FPLC: fast phase liquid chromatography

FRAP: fluorescence recovery after photobleaching

GFP: green fluorescent protein

GR: glucocorticoid receptor

HATs: histone acetyl transferases

HDACs: histone deacetylases

HMGB: high mobility group box

HOX: homeobox genes

IAA: iodoacetamide

ICAD: inhibitor of CAD

IEF: isoelectrophocusing

IgG: immunoglobulin G

IL-1: interleukin 1

LB: Luria-Bertani

LDH: lactate dehydrogenase

LEF-1: lymphocyte enhancer factor 1

LPS: lipopolysaccaride

MAPK: mitogen activated protein kinase

MEL: murine erythroleukemia cells

MHC: major histocompatibility complexes

MIF: migration inhibitor factor

MIP2: macrophage-inflammatory-protein-2

MMP: metalloproteases

MPO: myeloperoxidase

NF1: nuclear factor 1

NF-kB: nuclear factor-kB

NHP6: non-histone proteins 6

NMR: nuclear magnetic resonance

NP-40: nonidet P-40

OCT: octamer binding protein

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PCNA: proliferating cells nuclear antigens

PFA: paraformaldehyde

PKC: protein kinase C

PMSF: phenylmethylsulphonylfluorideliuos

RAG: recombination activating genes

RAGE: receptor for advanced glycation end-products

RSMC: rat smooth muscle cells

RSS: recombination Signal Sequences

SDS: sodium dodecyl sulphate

SDS-PAGE: SDS polyacrylamide gel electrophoresis

SRY: sex-determining region Y

TBP: TATA binding protein

TFII: transcription factor II

TLR: toll-like receptor

TNF-α: tumor necrosis factor-α

TSA: trichostatin A

TUNEL: TdT-mediated dUTP nick-end labelling

Wt: wild type

SUMMARY

High Mobility Group 1 (HMGB1) is an abundant and ubiquitous chromatin protein present in all vertebrate nuclei. It is endowed with a DNA bending activity which makes it act as an architectural chromatin factor: though it has no sequence specificity, it is recruited by several DNA-binding proteins to bend the DNA, thereby promoting the formation of multiprotein complexes on specific DNA targets.

Surprisingly, recent data have established that beyond its intranuclear function, HMGB1 also has a pivotal function outside of the cell, as an extracellular signal. A receptor for HMGB1 exists (RAGE, receptor for advanced glycation endproducts), and upon binding to the receptor, HMGB1 acts as a potent mediator of inflammation and cell migration.

My thesis work aimed to understand how the intracellular and the extracellular roles of HMGB1 are linked to each other, and starting from the analysis of the chromatin binding properties of HMGB1, it culminates with the proposal that the passive release of this abundant chromatin protein by dead cells may represent a "danger signal" which activates the immune system.

I used a GFP-fused HMGB1 in combination with photobleaching techniques to characterize the binding of the protein to chromatin. I found that the protein has differential chromatin binding properties in living, apoptotic, and necrotic cells. In living cells, HMGB1 exchanges rapidly between the soluble and the chromatin-bound states both in mitosis and in interphase. Fluorescence recovery after photobleaching (FRAP) and Fluorescence loss in photobleaching (FLIP) experiments have shown that HMGB1 moves very rapidly within the nucleus and binds to chromatin transiently, with extremely fast dissociation/association kinetics. In necrotic cells, HMGB1 is not

associated with chromatin and is rapidly leaked from chromatin into the extracellular medium as soon as membrane integrity is lost. In apoptotic cells, HMGB1 is retained in the nucleus because it binds non-covalently, but stably, to chromatin. FRAP and FLIP analysis have shown that in apoptosis HMGB1 is almost immobile and sticks to chromatin, and even late apoptotic cells, undergoing autolysis, do not release the protein.

In order to elucidate the molecular mechanism underlying the stable association of HMGB1 with chromatin in apoptosis, I checked for possible post-translational modifications of the protein in apoptotic cells. By 2D gel analysis, no difference in the pI or in the molecular weight of HMGB1 was observed comparing living and apoptotic cells, indicating that the protein may not be differentially modified in apoptosis. Instead, the causes of HMGB1 binding to chromatin are likely to be chemical and structural alterations in chromatin that create stable binding sites for HMGB1. In particular, histone H4 is deacetylated during apoptosis, and the inhibition of this deacetylation by tricostatin A treatment abolishes HMGB1 binding to chromatin.

The differential binding of HMGB1 to chromatin in necrotic and apoptotic cells is relevant with reference to the role of HMGB1 in promoting inflammation: necrosis is an unprogrammed mode of cell death and the primary response to necrosis is inflammation; in contrast, apoptosis is a physiological and programmed process and apoptotic cells only need physical removal and do not cause inflammatory response. We made the hypothesis that HMGB1 could be used by proinflammatory cells as a signal to distinguish necrosis and apoptosis. Indeed, necrotic cells lacking HMGB1 are rather ineffective in activating proinflammatory cells, and injections of anti-HMGB1 antibodies in wt mice reduce inflammatory responses induced by liver necrosis, indicating that HMGB1 is necessary to promote the inflammatory response to necrosis.

Moreover, if HMGB1 is detached from apoptotic chromatin by TSA treatment, also apoptotic cells become proinflammatory.

The conclusion deriving from this work suggests that the retention of HMGB1 by apoptotic cells might be a mechanism to prevent unwanted inflammatory reactions in response to programmed cell death: necrotic cells release HMGB1, thus informing the neighbour cells of their traumatic demise, and promoting inflammation; cells undergoing apoptosis, instead, withhold the signal, retaining HMGB1 tightly bound to chromatin, and they avert in this way inflammatory responses.

1 INTRODUCTION

1.1 HMGB proteins (High Mobility Group Box)

The HMGB family comprises in mammals the three proteins HMGB1 (previously, HMG1), HMGB2 (previously, HMG2) and HMGB3 (previously, HMG4 or HMG2b) (Bustin, 2001). HMGB proteins are very abundant, non-histone chromatin proteins present in the nucleus of all eukaryotic cells. They are small proteins (~ 25 KDa) characterised by high mobility in denaturing SDS polyacrylamide gels, hence the acronym High Mobility Group (Bustin et al., 1990).

HMGB1 is the archetypal member of the group and gives the name to the family. It is ubiquitous and only 10 times less abundant than core histones, counting about one million molecules per typical mammalian cell. The expression of the other two family members is more restricted: HMGB3 is only expressed to a significant amount during embryogenesis (Vaccari et al., 1998); HMGB2 is widely expressed during embryonic development, but restricted mainly to lymphoid organs and testis in the adult mouse (Ronfani et al., 2001).

The structure of these three proteins is highly conserved (**Fig. 1**) (more than 80% amino acid identity), and their biochemical properties are so far indistinguishable. HMGBs are composed of three different domains. The two homologous DNA binding domains, HMG box A and B, are each around 75 amino acids in length. The C-terminal domain is highly negatively charged, consisting of a continuous stretch of glutamate or aspartate residues, and is longest in HMGB1 and shortest in HMGB3 (reviewed in Bianchi and Beltrame, 2000; Bustin, 1999).

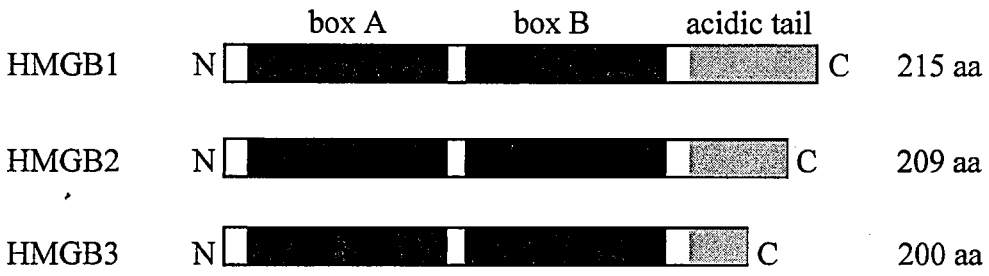

Fig. 1 Structure of HMGB proteins. All HMGBs possess two DNA binding domains, HMG box A and B (black boxes), and an unstructured acidic tail of variable length at the C-terminus (grey box).

Putative counterparts of HMGBs are present in *Saccharomyces cerevisiae* (non-histone proteins 6A and 6B, NHP6A and NHP6B) (Kolodrubetz and Burgum, 1990) and *Drosophila melanogaster* (HMG-D and HMG-Z) (Wagner et al., 1992), as well as in plants (**Fig. 2A**). Single HMG-box domains, with no acidic tail, characterize also an increasing number of mammalian sequence-specific transcription factors, such as the sex determining factor SRY (sex-determining region Y) (Goodfellow and Lovell-Badge, 1993) and the lymphocyte enhancer factor 1 (LEF-1) (Verbeek et al., 1995).

1.2 The HMG-box domain

All the known biochemical and functional properties of HMGBs are specified by their HMG-boxes. The HMG-box motif is a stretch of about 75 residues, with a net positive charge and rich in aromatic residues and prolines (Bianchi et al., 1992). No amino acid is absolutely conserved in all known HMG-boxes, and only three residues have conservative substitutions.

The three-dimensional structures of several HMG-boxes have been resolved by means of either NMR or crystallography, showing that the global fold of the domain is well conserved (Read et al., 1993; Hardman et al., 1995; Weir et al., 1993; Jones et al., 1994; Werner et al., 1995). The HMG-box of HMGB1 consists of three α-helical segments forming an L-shaped structure stabilised by a hydrophobic core. The shorter arm of the "L" consists of helices I and II and the longer arm is composed of the extended N-terminal section packed against the C-terminal region, helix III (**Fig. 2B,C**).

Despite having a broadly similar fold, the structures of the A and B domains of HMGB1 differ significantly in the relative disposition of helices I and II and in the trajectory of the helix I–II loop. In box A, helix I is essentially straight whereas in box B it is bent, and the loop between helices I and II is longer in box A than in box B.

The HMG boxes of HMGB1 are highly similar to the HMG domains of *S. cerevisiae* NHP6A and *Drosophila* HMG-D and, to a lesser extent, to the sequence-specific (e.g. SRY and LEF-1) HMG-boxes.

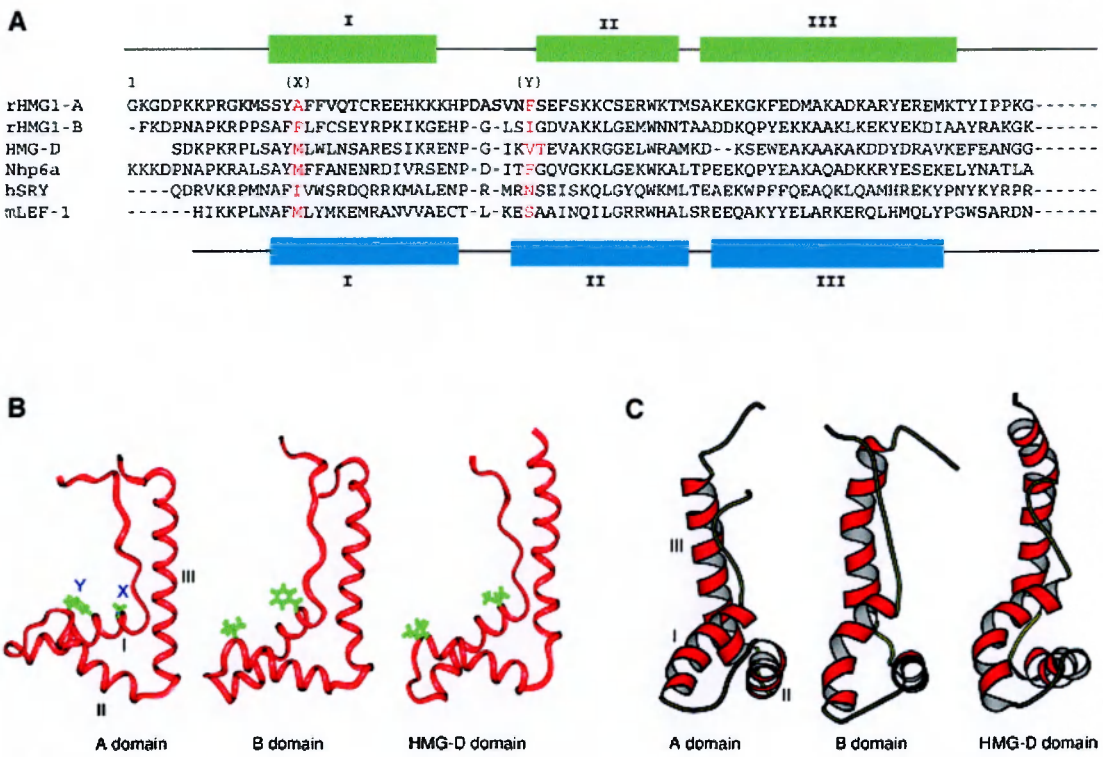

Fig. 2 Comparison between amino acid sequences and folds of different HMG boxes.

A. Residues of non-sequence-specific and sequence-specific HMG boxes intercalating into the DNA minor groove (marked in red and indicated as X and Y) are localised with respect to the α-helices determined by NMR spectroscopy for HMGB1 box A (shown in green above the sequence) and the sequence-specific LEF-1 HMG box (shown in blue below the sequence). Prefixes r, h and m signify rat, human and mouse, respectively.

B. Location of residues at X and Y in the 3D structures of HMGB1 box A, HMGB1 box B (inferred intercalations) and the HMG-box of HMG-D.

C. Solution structures of the A and B HMG boxes of HMGB1 and of the HMG box of HMG-D, determined by NMR spectroscopy (Hardman et al., 1995; Weir et al., 1993; Jones et al., 1994). The structures are oriented to show the differences in the relative disposition of helices I and II between the different domains.

The main feature of the HMG-boxes of HMGB1 is the ability to both recognise and produce distorted structures in DNA. HMGB1 binds rather inefficiently to linear DNA, with no preference for any nucleotide sequence. It shows, on the contrary, high affinity and selectivity to structured DNA, such as 4-way junctions, cisplatin-DNA adducts, and kinked DNA (Bianchi et al., 1989, 1992). The binding of HMGB1 to DNA is therefore sequence-independent, but structure-specific. Beyond the ability to recognise distorted structures, HMGB1 can also introduce them in the double helix, thanks to a DNA bending activity. In spite of its low affinity for linear DNA, when present at high concentrations, HMGB1 is capable of inducing DNA bends upon binding, as revealed by ring closure of short DNA fragments (Pil et al., 1993).

As yet, no structure of a complex between HMGB1 and linear DNA has been solved. However, the similarity with other HMG-boxes, for which structural data are available (Werner et al., 1995; Murphy et al., 1999), is revealing with respect to the mechanism underlying HMGB1-mediated DNA bending. The DNA-binding surface of the HMG box domain has a hydrophobic surface that conforms to a wide, shallow minor groove. In the centre of this surface, a hydrophobic wedge, usually consisting of four spatially close residues, inserts deep into the minor groove. This partial intercalation introduces a kink into the bound DNA, enhancing the more uniform bend associated with the widening of the minor groove (**Fig. 3A**). The only resolved complex between HMGB1 and a DNA substrate is the X-ray structure of the rat HMG-box A domain complexed to cisplatin-modified DNA (**Fig. 3B**) (Ohndorf et al., 1999). Cisplatin is a drug that damages DNA, creating intrastrand crosslinks between adjacent purines (Pil and Lippard, 1992). These crosslinks bend and unwind the duplex, generating a structure which HMGB1 binds with high affinity, without inducing any

further distortion. The analysis of the complex revealed which amino acids are mainly involved in contacts with DNA: particularly important appeared to be a phenylalanine residue in helix II (see **Fig. 2A,B**), which intercalates into the kinked platinated GpG site, and whose substitution dramatically reduces the binding of the domain. Nevertheless, the cisplatin-induced intrastrand crosslinks alter the contact surface for the HMG box on DNA, compared to a linear molecule, and indeed significant differences are observed with respect to LEF1- and SRY-DNA complexes. These proteins induce in DNA a bend similar to the one formed at the kinked platinated site, but the bend locus in this case is near the centre of the protein-binding site. In contrast, the binding surface of HMGB1 box A extends exclusively to the 3' side of the bend, when the platinated strand is used as a reference, and it covers five base-pair steps (**Fig. 3B**). Thus, the relationship between the DNA bend and the protein-binding site is slightly altered by the cisplatin-induced intrastrand crosslinks. Taking this into account, and considering that the general fold of all HMG boxes is well conserved, a complex between the HMG box of HMGB1 and a linear DNA is likely very similar to those formed by the sequence-specific HMG boxes (**Fig. 3C**).

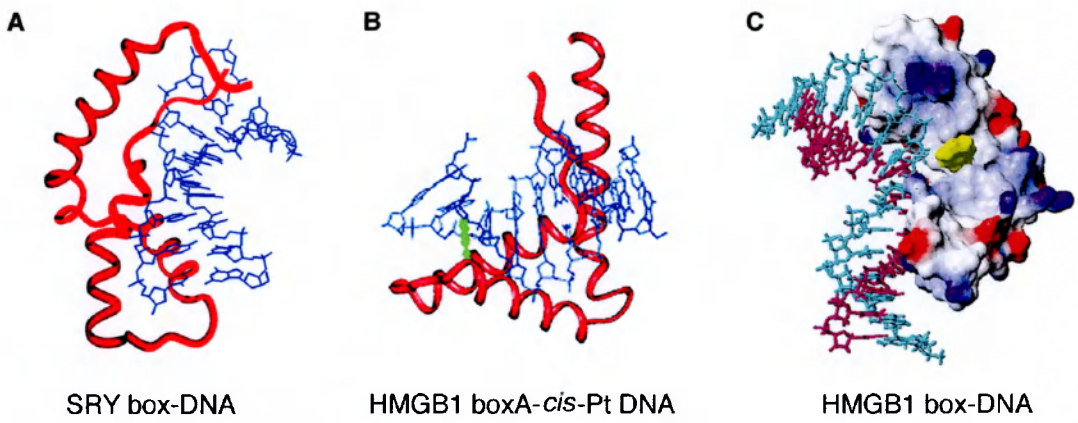

Fig. 3 Structures of HMG box-DNA complexes.

A. Structure of the complex between the HMG box (red) of human SRY and the octanucleotide GCACAAAC (blue). The DNA is significantly distorted: the minor groove is widened considerably to accommodate the extended stretch of the HMG box; the planes of the bases are tilted, and the double helix is unwound and bent. The contact between the HMG box and the DNA occurs over a surprisingly large area and with a very tight fit; thus, the energetic cost of distorting the DNA is more than compensated by the chemical energy freed by the protein-DNA interaction (Werner et al., 1995).

B. The complex of the HMGB1 box A with cis-platinated DNA (X-ray crystal structure) (Ohndorf et al., 1999). Remarkably, in this structure the orientation of the intercalating Phe group differs from that in the solution structure of the free domain shown in Fig. 2B.

C. Model of HMGB1 box B positioned on 15 bp of DNA. The model is derived from the high similarity between sequence-specific HMG boxes and the HMGB boxes of HMGB1. The electrostatic surface (blue is positive charge; red is negative charge) is shown with the intercalating phenylalanine residue in yellow. DNA strands are shown in magenta and cyan (Wolffe, 1999).

1.3 HMGB proteins as architectural regulators

Many studies have been performed over the last few years in the attempt to correlate the characteristic DNA-binding properties of the HMG-box domain to the biological roles of the abundant HMGB proteins. One proposed general function of these proteins is to overcome the barrier imposed by the axial rigidity of DNA and thereby promote the formation of complex nucleoprotein assemblies containing tightly bent DNA (Crothers, 1993). Such a role probably requires that the bend is precisely placed; a displacement of the binding site by even 1 bp would alter the trajectory of the bent DNA by 30–36°. Because DNA-sequence recognition by the abundant HMGB proteins lacks selectivity, the precise targeting of these proteins must depend on the presence of other sequence-specific DNA-binding proteins in the complex. This indeed appears to be the case.

HMGB1 and HMGB2 have been implicated, from several *in vitro* assays, in both the activation and repression of transcription. A recurring theme is the enhancement of the binding of various transcription factors [e.g. Oct-1 and 2 (Zwilling et al., 1995), HoxD9 (Zappavigna et al., 1996), p53 (Jayaraman et al., 1998), Rel proteins (Decoville et al., 2000), and steroid hormone receptors (Boonyaratanakornkit et al., 1998)] to their cognate DNA binding sites. In most of these cases, the interaction of the HMGB protein with a transcription factor has been detected *in vitro* even in the absence of DNA, and could presumably serve as the mechanism for recruitment of HMGB1,2 to particular DNA sites (**Fig. 4**). In turn, HMGB proteins bound to their partners increase the protein surface contacting DNA, from both the major and the minor groove, thereby achieving high affinity interactions. In some cases, transfection experiments have also proved functional interactions *in vivo*. The demonstrated interactions *in vitro* are between HMGB1 and/or 2 and a single transcription factor. However, it is probable that *in vivo*,

in a natural regulatory context, the DNA bending by HMGB1 or 2 may allow the recruitment of a second transcription factor to the complex, generating a complex network of interactions (**Fig. 4**).

In vitro interactions between HMGB proteins and the basal transcription machinery have also been reported. Human HMGB1 binds to the TATA-box binding protein (TBP) and interferes with the normal binding of TFIIB in the pre-initiation complex, thereby inhibiting TBP function (Ge and Roeder, 1994; Sutrias-Grau et al., 1999); both HMGB1 and TFIIB independently enhance binding of TBP to TATA-box DNA. By contrast, HMGB2 has been reported to stabilize and activate the TBP-containing TFIID–TFIIA complex bound to promoter DNA (Shykind et al., 1995).

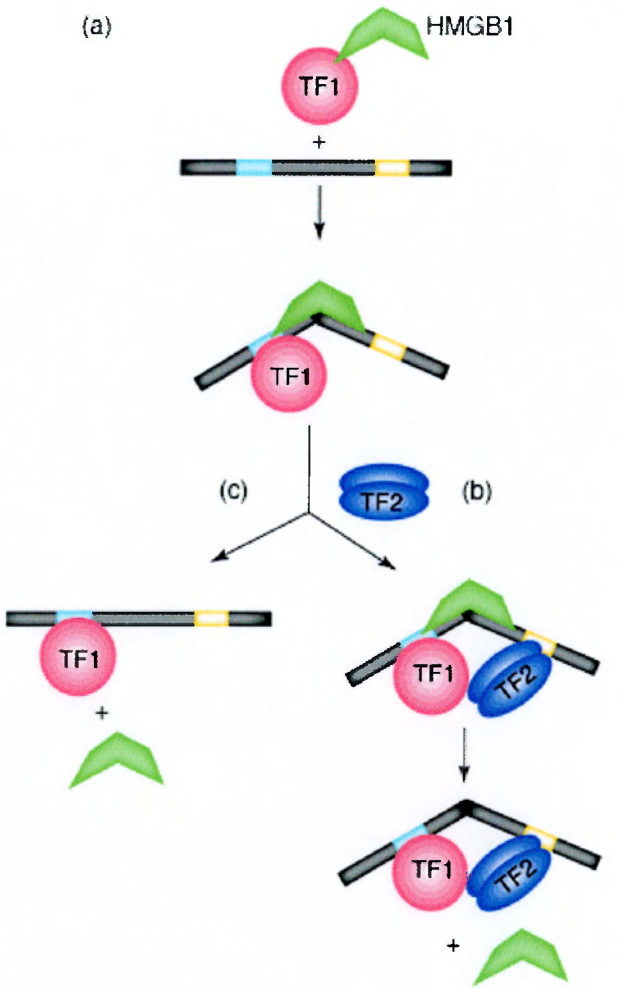

Fig. 4. A possible chaperone role for HMGB1 in facilitation of transcription-factor binding. (a) Recruitment of HMGB1 (green) to specific DNA sites through interaction with a sequence-specific transcription factor (TF1; red). Formation of a ternary complex that is probably unstable, but has been detected and/or inferred in some cases. In this ternary complex, HMGB1 might bend the DNA, thus providing the potential for the recruitment of additional DNA-binding proteins (TF2; purple) to the complex (b). If no additional protein is recruited, HMGB1 could dissociate from the complex, leaving the transcription factor stably bound (c) [in this diagram, the final DNA is shown straight (as in the case of Oct-1 binding), but this is not necessarily the case; for example, the progesterone receptor bends its DNA in the binary complex]. Only a single HMG box is shown because the separate A and B HMG boxes of HMGB1 have been shown to facilitate binding of various transcription factors *in vitro*.

HMGB proteins exert their architectural function also in other nuclear processes involving manipulation of DNA structure. The best example is observed in lymphocytes, during V(D)J recombination, a complex process which allows the generation of a nearly infinite number of antibodies and T Cell Receptors. In developing T and B lymphocytes, antigen receptor genes are somatically assembled from several V (variable), D (diversity), and J (joining) DNA segments, in a process mediated by two lymphoid-specific enzymes, RAG1 and RAG2 (products of Recombination Activating Genes 1-2) (Schatz et al., 1989). These proteins bind specifically to the Recombination Signal Sequences (RSS), regions flanking each antigen receptor segment, which direct the site of rearrangement. Upon binding, the RAG1/2 complex cleaves the DNA, producing a hairpin intermediate with nucleotide overhangs. In the second step of the process, while RAG1/2 remain stably bound to the RSS, the free ends generated by the cleavage are linked to each other by DNA repair activities. It has been shown that HMGB1 and HMGB2 are recruited by RAG1/2 to the RSS, and functionally interact with the complex, facilitating V(D)J recombination (Aidinis et al., 1999). Also in this case, a physical interaction between HMGB1,2 and RAG1/2 enhances the binding of the enzymes to the RSS. The RAG1/2 complex possesses an intrinsic DNA bending activity, and upon binding, it alters the structure of the RSS site even in the absence of HMGB1,2. However, the DNA bending induced by RAG1/2 is very inefficient unless assisted by HMGB1,2, suggesting a crucial contribution of HMGB proteins in stabilising the complex between RAG1,2 and the bent RSS (Aidinis et al., 1999).

The picture emerging from all these data point to HMGB proteins as important and versatile architectural chromatin components, required for the formation and the

stabilisation of functional nucleoprotein complexes. This function is sustained by their peculiar DNA binding properties and their ability to modulate the structure of DNA, which guarantee a correct three-dimensional assembly of DNA binding proteins on bent DNA.

1.4 Phenotype of HMGB1- and HMGB2- deficient mice

Mice lacking either HMGB1 or HMGB2 were produced in our lab, by conventional knock-out, in order to study the function of the proteins *in vivo* (Calogero et al., 1999, Ronfani et al., 2001). The most dramatic phenotype is displayed by *Hmgb1*$^{-/-}$ mice, which are born, but die within the first day of life as a result of severe hypoglycaemia (**Fig. 5A**) (Calogero et al., 1999). While the level of glucose is decreased in the blood of *Hmgb1*$^{-/-}$ animals, abundant glycogen is present in their liver (**Fig. 5B**), but is not metabolised. Indeed, intraperitoneal injection of glucose in *Hmgb1*$^{-/-}$ newborns permits the survival of some animals, even though the rescued mice remain very sick, with several anatomical abnormalities (**Fig. 5C**).

At the cellular level, *Hmgb1*$^{-/-}$ mice have defects in steroid hormone-dependent gene expression. Fibroblasts from knock-out mice transiently transfected with a reporter plasmid containing a GR responsive element respond weakly to the glucocorticoid analogue dexamethasone, compared to wt fibroblasts (**Fig. 5D**). Moreover, CD4^{+} CD8^{+} T cells from *Hmgb1*$^{-/-}$ mice are partially resistant to dexamethasone exposure, while wt CD4^{+} CD8^{+} cells undergo massive apoptosis upon treatment (Calogero et al., 1999). All these findings are consistent with the proposal that HMGB1 helps steroid hormone receptors in controlling their target genes.

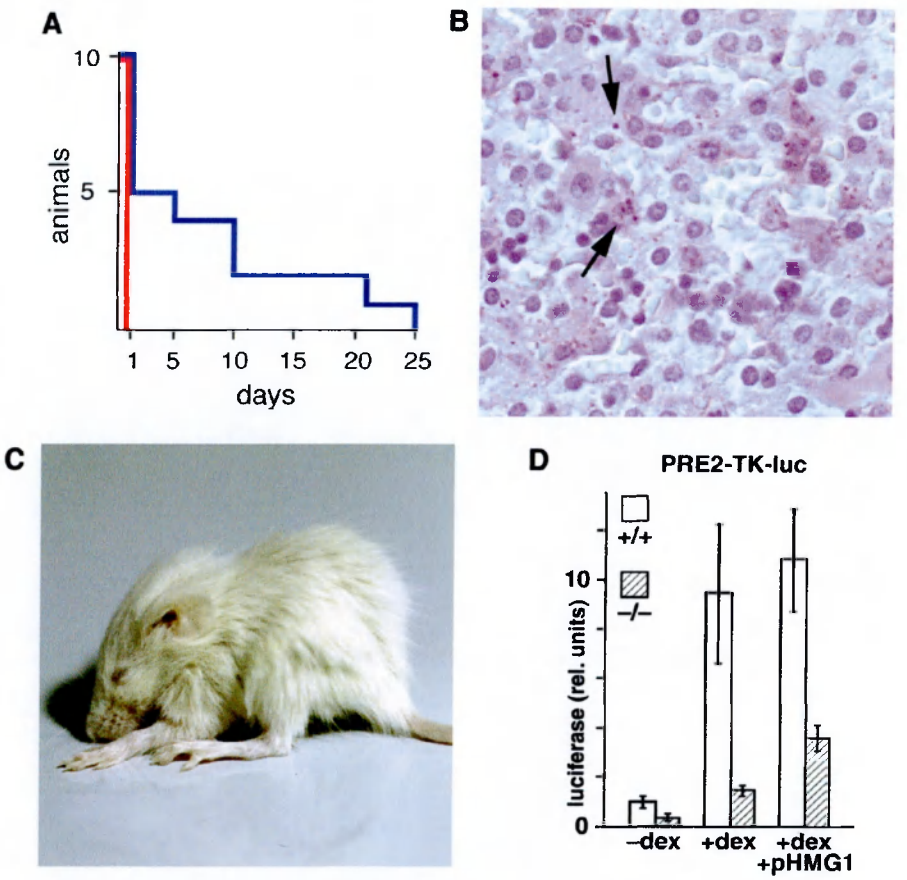

Fig. 5 *Hmgb1*$^{-/-}$ mice develop neonatal hypoglycaemia.

A. *Hmgb1*$^{-/-}$ mice die within the first day of life but can survive if given glucose parenterally. Intraperitoneal glucose injections were administered to 10 *Hmgb1*$^{-/-}$ newborns during the first days after birth. Survival is indicated by a blue line. Ten *Hmgb1*$^{-/-}$ control mice, injected with saline solution, all died within day 1 (red line), similar to untreated *Hmgb1*$^{-/-}$ mice.

B. Periodic acid-Schiff (PAS) staining for glycogen (magenta) in livers from *Hmgb1*$^{-/-}$ mice are shown. Despite very low blood glucose concentrations, hepatocytes of *Hmgb1*$^{-/-}$ mice do not completely mobilize glycogen (arrows, glycogen granules).

C. Phenotype of an *Hmgb1*$^{-/-}$ spontaneous survivor at day 25. Spontaneous survivors of the mixed 129Sv/CD1 genetic background are very similar to the most successfully glucose-rescued *Hmgb1*$^{-/-}$ mice of 129Sv/BALB-c background: they have a very reduced size, but respond positively to basic neurological tests. All spontaneous or glucose-treated surviving animals have sealed eyelids, arched backs, long hind paws and abnormal gait.

D. The absence of Hmgb1 reduces the activity of GR in transfection assays. The expression of a GR-controlled reporter is reduced in *Hmgb1*$^{-/-}$ fibroblast cell lines exposed to dexamethasone.

Unexpectedly, however, no defects in V(D)J recombination were found: even though the *in vitro* data implicate HMGB1 in the process, the newborn mice have a normal serum abundance of immunoglobulins, and normal numbers of mature single-positive $CD4^+$, and $CD8^+$ T cells expressing a complete Vß repertoire in the thymus (Calogero et al., 1999). Moreover, in spite of the cooperation between HMGB1 and HOX proteins and the basal transcription machinery, the gene regulation necessary to generate a neonatal animal with fully differentiated tissues is unimpaired in mutant mice.

The most plausible explanation for the lack of defects in the V(D)J products and in the body plan of the *Hmgb1*$^{-/-}$ mice is that HMGB2 could compensate for the absence of HMGB1 in the interaction with RAG1/2 and HOX proteins. HMGB2 is widely expressed during embryonic development, when HOX proteins exert their function, and even after birth it is particularly concentrated in the thymus, which is the site of V(D)J recombination. Therefore, even in the absence of HMGB1, HOX proteins and RAG1/2 could find a suitable partner. By contrast HMGB2 is completely absent from liver in the adult, and this might explain the defects in the GR-mediated functions and the hypoglycaemic phenotype of the *Hmgb1*$^{-/-}$ mice.

In order to test the possibility that HMGB2 might provide partial redundancy to HMGB1, *Hmgb2*$^{-/-}$ mice were also generated, with the perspective to generate double *Hmgb1*$^{-/-}$ *Hmgb2*$^{-/-}$ mutants. Mice lacking HMGB2 are apparently healthy, and only show a reduced fertility in males. Increased apoptosis of germ cells in seminiferous tubules and production of defective spermatozoa appeared to underlie this defect (**Fig. 6**) (Ronfani et al., 2001). This phenotype correlates with the observation that HMGB2 is highly abundant in the testis, even more than HMGB1.

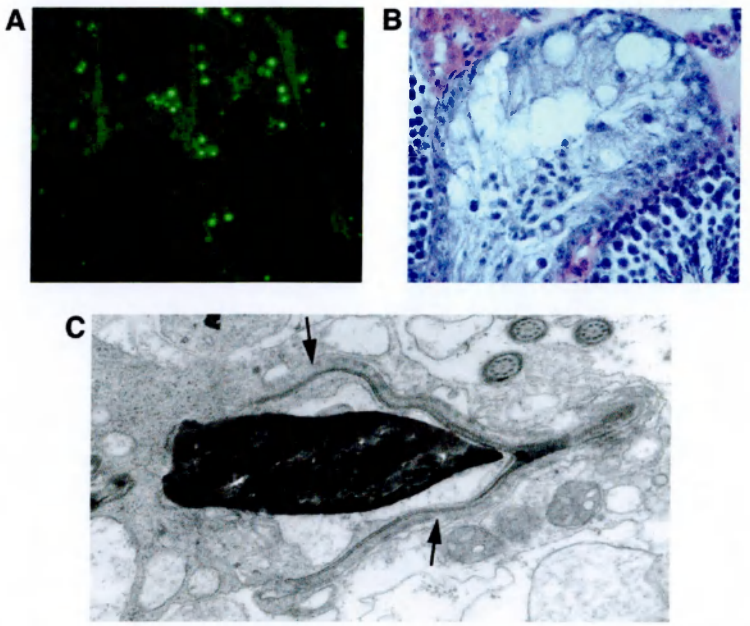

Fig. 6 Abnormalities in the testis of *Hmgb2*$^{-/-}$ mice.

A. TUNEL staining for apoptotic cells in testis seminiferous tubules of *Hmgb2*$^{-/-}$ mice, showing the high content of dead cells.

B. Haematoxylin-eosin stained sections of testis from a 163-day *Hmgb2*$^{-/-}$ mouse. The regular periphery-to-lumen succession of spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa is lost, cells are separated by gaps, and degenerated Sertoli cells with large vacuoles are present.

C. Electron micrographs of an elongated spermatid where the acrosome (arrows) is detached from the nucleus, impairing its function.

Taken together, the data obtained from HMGB1- and HMGB2- deficient mice suggest that HMGB1 and HMGB2 are indeed functionally equivalent, and a minimum total amount of HMGB1 plus HMGB2 is required at all times. The lack of HMGB1 is phenotypically more noticeable because HMGB1 is ubiquitous and usually much more abundant than HMGB2. In contrast, as HMGB2 has a more restricted pattern of

expression, and in most cases HMGB1 can compensate for the loss of HMGB2, the defects in *Hmgb2*$^{-/-}$ mice are less severe and more localised. In order to definitively prove the redundancy of HMGB proteins, we are currently breeding HMGB1 and 2 knockouts, to generate double mutants. So far, no double knock-out has been found at embryonic day 14 ($p < 0.05$). This result implies that HMGB1 and HMGB2 have indeed similar functions and a sufficient amount of HMGBs is required for embryonic development.

1.5 HMGB1 and chromatin

The most surprising finding derived from *Hmgb1*$^{-/-}$ mice is that HMGB1 is not essential for the life of cells, in spite of its abundance and evolutionary conservation. This observation impacts on the proposed role of HMGB1 as structural protein involved in packaging DNA into chromatin.

For many years, HMGB proteins have been considered to cooperate with histones in determining chromosome architecture. Both protein families appeared to serve the function of wrapping DNA into arrays of repetitive modules, thereby ensuring the formation of organised high-order structures (Wisniewski and Grossbach, 1996; Zlatanova et al., 1999). On average, a typical cell nucleus contains one molecule of HMGB1 every 10-20 nucleosomal particles, and a role for the protein in promoting the formation of nucleosomes has been suggested (Travers et al., 1994). In addition, HMGB1 has been proposed to stabilize nucleosomes by binding at the DNA crossover site (Lilley, 1992), with a function similar to that of histone H1. Indeed, HMGB1 and histone H1 share several properties. *Xenopus* HMGB1 (xHMG1) binds to nucleosomes *in vitro* in much the same way as histone H1 (Nightingale et al., 1996; Ura et al., 1996);

the *Drosophila* HMGB1-like protein HMG-D is a structural component of condensed metaphase chromosomes (Ner and Travers, 1994); histone H1 also binds to four-way junctions (Varga-Weisz et al., 1993). These observations suggested that HMGB1 and H1 could possess similar overall properties but specifically adapted to perform subtly different structural functions.

The first data in conflict with this model were the finding that the association of HMGB1 with chromatin is markedly different from that of histone H1 (Falciola et al., 1994). If cells are treated with NP-40, a non-ionic detergent which permeabilises plasma and nuclear membranes, nuclear proteins not tightly bound to DNA leak into the extracellular medium, whereas stable components of chromatin remain anchored to the nuclear remnants (**Fig. 7A**). Permeabilised cells, both in mitosis and in interphase, release the vast majority of HMGB1, indicating that the protein is not a structural component of either condensed or decondensed chromatin (**Fig. 7B**). On the contrary, histone H1 remains firmly associated with DNA, as well as the core histones and other chromatin binding proteins. Moreover, immunofluorescence analysis showed that while in interphase HMGB1 is uniformly distributed in the nucleoplasm, like histone H1, in mitosis, contrary to H1, it is totally displaced from chromosomes (Falciola et al., 1994). In partial contrast with these observations, however, *in vitro* HMGB1 appeared to bind to reconstituted nucleosomes with high affinity, forming complexes sufficiently stable to survive band-shift assays (Falciola et al., 1994). Thus, HMGB1 can bind to nucleosomes, but *in vivo* its association with chromatin is quite loose. Taken together, these findings disproved a structural role of HMGB1 in packaging bulk DNA, and rather suggested that the protein may establish only transient interactions with chromatin.

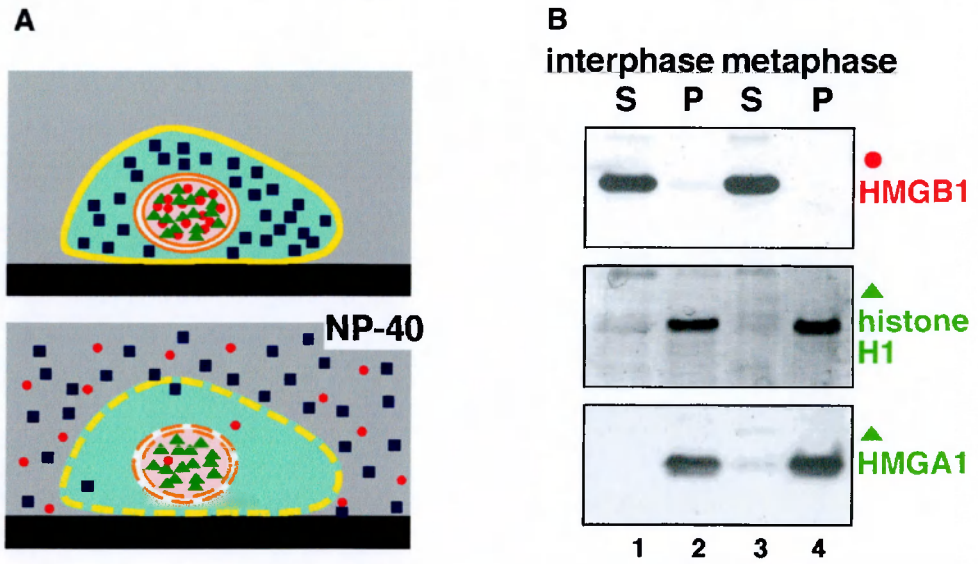

Fig. 7 HMGB1 is not stably associated to chromatin.

A. The cartoon shows what happens after NP-40 addition: both cellular and nuclear membranes are permeabilised and soluble proteins (blue squares and red circles) leak out from nucleus and cytoplasm. Thus, nuclear proteins not stably bound to DNA (red circles) are released in the extracellular milieu, whereas histones and other stable component of chromatin (green triangles) are retained in the nucleus.

B. After permeabilisation, the buffer bathing the cells (lanes 1 and 3; S: supernatant) and the cellular remnants, containing chromatin associated proteins (lanes 2 and 4; P: pellet) can be analysed by Western blot with different antibodies, to distinguish between stable components of chromatin and proteins that interact with DNA only transiently. While histone H1 and HMGA1 are found in the pellet, bound to DNA, HMGB1 is released outside the cells both in interphase and in mitosis.

The results described above indicated that HMGB1 and histone H1 do not have equivalent functions in differentiated mammalian cells, but did not rule out the possibility that HMGB1 might vicariate H1 when the linker histone is absent. This is what happens in *Xenopus* and *Drosophila*, where respectively xHMG1 and HMG-D

appear to substitute for histone H1 during embryogenesis (Dimitrov et al., 1994, 1993; Ner and Travers, 1994). Later studies addressed this problem, investigating the association of HMGB1 with chromatin in very early mouse development, a period when histone H1 is present at very low abundance (Clarke, 1992). Nonetheless, even in preimplantation embryos, the affinity of HMGB1 for chromatin appeared similar to that observed in somatic cells, and the protein did not remain associated with chromosomes during metaphase (Spada et al., 1998). This result indicated that HMGB1 has non-overlapping roles with the linker histone and does not compensate for its absence during mouse early embryogenesis.

1.6 A new field of investigation: HMGB1 is also an extracellular signal

In accordance with its role as an architectural component of chromatin, the localisation of HMGB1 is nuclear in most cell types. Surprisingly, recent data have established that beyond its intranuclear function, HMGB1 also has a pivotal function outside of the cell.

1.6.1 HMGB1 as a potent mediator of inflammation

Wang *et al.* (1999a) have recently identified HMGB1 as a late mediator of endotoxin lethality in mice. HMGB1 was found to be secreted by monocytes/macrophages stimulated by bacterial lipopolysaccaride (LPS, endotoxin), and to accumulate in the serum of the mice, mediating septic shock.

LPS is a component of all Gram-negative bacteria. It is toxic to mammals because it activates the innate immune system (macrophages and neutrophils) to secrete proinflammatory cytokines, such as TNF-α, interleukin 1 (IL-1), and macrophage

migration inhibitory factor (MIF) (Tracey and Cerami, 1993; Bernhagen et al., 1993). These factors drive the acute phase response and mediate the development of shock and tissue injury. In recent years, therapeutic strategies for sepsis have attempted to modulate the excessive inflammatory response by inhibiting cytokines released by macrophages with specific antibodies. However, a major difficulty in targeting cytokine activities derives from the fact that most proinflammatory mediators are released early in septic shock, and inhibitors should therefore be administered immediately. In order to broaden the therapeutic window for sepsis, Wang *et al.* initiated a search for a putative macrophage-derived mediator of lethality that appears relatively late after the onset of endotoxemia. This study culminated in the identification of HMGB1 as the proposed late mediator of LPS lethality. The serum concentration of HMGB1 significantly increases 16-32 hours after LPS administration in mice. Administration of anti-HMGB1 antibodies attenuated LPS-induced endotoxemia, and conversely, injection of HMGB1 caused toxic shock. Moreover, septic patients showed increased serum levels of HMGB1, which correlated with the severity of the infection (Wang et al., 1999a).

The source of extracellular HMGB1 are activated monocytes/macrophages. Macrophage-like RAW 264.7 cells release the protein 18 hours after stimulation with LPS; confirmation of the inducible nature of HMGB1 release was obtained also in murine primary peritoneal macrophages, and in human primary peripheral blood mononuclear cells (Wang et al., 1999b). Pituicytes, which provide an important link between the immune and the neuroendocrine systems, also release HMGB1 in response to specific stimuli like TNF-α and IL-1, suggesting that HMGB1 participates in the regulation of neuroendocrine and immune responses to inflammatory processes (Wang et al., 1999b).

Subsequently, HMGB1 was also shown to cause acute lung inflammation when administered intratracheally (Abraham et al., 2000). Antibodies against HMGB1 decreased lung edema and neutrophil migration, whereas they did not reduce the levels of the other proinflammatory cytokines, such as TNF-α, IL-1β or macrophage-inflammatory-protein-2 (MIP2).

It is not clear so far how HMGB1 is secreted by monocytes and the other competent cell types: it possesses no signal peptide that would direct it to the endoplasmic reticulum (ER). This lack of a secretion leader peptide is a feature shared with a small number of other secreted proteins, like the cytokine IL-1β (Andrei et al., 1999). Studies in murine erythroleukemia (MEL) cells have shown that HMGB1 export does not involve the ER and the Golgi complex, but is promoted by intracellular Ca^{2+} increase and possibly by activation of a Ca-dependent PKC isoform (Passalacqua et al., 1997).

In any case, the first event required for HMGB1 secretion is nuclear export. We proved that HMGB1 is not confined in the nucleus, but rather constantly shuttles between the nucleus and cytoplasm (unpublished data). The meaning of this traffic is still not clear; however, every time the protein goes to the cytoplasm, an opportunity arises to enter the secretory pathway.

Once released, HMGB1 is able to activate several other cells involved in the immune response or inflammatory reactions, and can act as a cytokine itself (Andersson et al., 2000). HMGB1 activates monocytes to secrete a specific subset of proinflammatory cytokines, including TNF-α and IL-1. Stimulation occurs at the level of gene transcription, as cytokine mRNA levels increase after HMGB1 stimulation (Andersson et al., 2000). In comparison with the well-known inflammatory stimulus

LPS, HMGB1 causes a delayed and biphasic release of TNF-α, with a first peak at 3 hours, followed by a second peak at 8-10 hours after HMGB1 exposure.

In light of all these data, it is now clear that HMGB1 has a crucial role during phlogystic processes: it is secreted by macrophages in response to proinflammatory stimuli, and also itself provokes a delayed response, thereby prolonging and sustaining inflammation.

1.6.2 HMGB1 in differentiation processes

Before the discovery of the role of HMGB1 in inflammation, it had already been observed that several cells release the protein into their surroundings as part of their differentiation process. Murine erythroleukemia (MEL) cells, exposed to a chemical inducer (hexamethyl-enebisacetamide, HMBA), release HMGB1 (also called DEF for differentiation enhancing factor) into the medium. In turn, extracellular HMGB1 significantly increases the rate of MEL cell differentiation via specific binding to the external surface of the cell membrane (Passalacqua et al., 1997; Sparatore et al., 1996).

Likewise, HMGB1 is also secreted by neurons and participates in their differentiation process (Fages et al., 2000, and references therein). Some years ago, an extracellular, membrane-bound form of HMGB1 was purified from rat brain for its capability of mediating neurite outgrowth. The membrane bound form was named amphoterin because of its dipolar structure, but at the sequence level it is identical to HMGB1. Membrane HMGB1 has been implicated in enhancing neurite outgrowth in cerebral neurons during development, and in regenerating peripheral neurons by its binding to a cell surface proteoglycan, syndecan. Membrane HMGB1 is abundant in both neuronal cell bodies and in neurites in neuroblastoma cells, whereas in neurons and peripheral glial cells (Schwann cells) it is localised in non-nuclear compartments.

HMGB1 mRNA is developmentally regulated, and reduced significantly after the rapid perinatal growth phase of the rat brain. The molecular basis for cellular targeting of HMGB1 to membranes remains unclear.

HMGB1 can also be exported in the extracellular space by groups of cells which support the differentiation of other spatially associated cell types. Stimulated astrocytes release HMGB1, which then induces LAN5 neuroblastoma cells to differentiate (Passalacqua et al., 1998). Human promyelocytic HL60 cells, although not secreting HMGB1 themselves, show an accelerated differentiation when exposed to extracellular HMGB1 (Sparatore et al., 1996).

In all these cases, the release of HMGB1 into the extracellular space is well controlled and local: HMGB1 is either secreted in an autocrine manner, or in a paracrine manner by closely associated cells. Remarkably, this spatially restricted secretion of HMGB1 does not seem to trigger an inflammatory response.

1.6.3 HMGB1 mediates cell migration and metastasis

HMGB1 can also promote cell migration. Cells migrate in response to extracellular stimuli, channelled through specific signal transduction pathways, that ultimately elicit cytoskeletal remodelling. We showed in our lab that vascular smooth muscle cells respond to extracellular HMGB1 (Degryse et al., 2001). Cells exposed to HMGB1 undergo rapid and transient changes of cell shape, and actin cytoskeleton reorganization, leading to an elongated polarized morphology typical of motile cells.

Even more strikingly, Taguchi *et al.* (2000) showed that HMGB1 is also involved in tumour growth and metastasis: administration of anti-HMGB1 antibodies suppressed metastasis formation by Lewis lung tumour cells implanted under the skin of recipient mice. In this context, HMGB1 promotes both migration and proliferation of tumor cells.

In vivo, cell migration depends to a large extent on the capability of a cell to invade the surrounding tissue, for which the activation of extracellular proteases is required. HMGB1 appears to play a role here as well: it binds to several components of the plasminogen activation system and enhances the activation of t-PA (Parkkinen and Rauvala, 1991). Moreover, Taguchi *et al.* (2000) showed HMGB1-elicited activation of metalloproteases MMP-2 and MMP-9, which are downstream targets of the plasmin activation cascade. While it enables the degradation of the extracellular matrix, the activation of proteases also leads to degradation of HMGB1, and this might serve as a feedback mechanism.

1.6.4 Signalling mechanisms

The signalling mechanisms by which HMGB1 activates cells to respond are incompletely understood. HMGB1 is rather "sticky", and binds to many different molecules on the cell surface: heparin, proteoglycans, but also sulfoglycolipids and phospholipids (Bianchi, 1988; Degryse et al., 2001; Rouhiainen et al., 2001). This could be a mechanism to restrict the diffusion of extracellular HMGB1, and thereby keep the effect of this potentially dangerous molecule local. HMGB1 can also be taken up by the cell in an as yet unidentified manner, and promote the co-uptake of DNA. This property was therefore used to transfect cells (Mistry et al., 1997). It is however unclear whether binding to cell surface glycans and cellular uptake are causally related.

However, at least one high-affinity receptor for HMGB1 exists: RAGE (receptor for advanced glycation endproducts) (Hori et al., 1995). RAGE belongs to the immunoglobulin superfamily and binds a variety of ligands: the glycated proteins that are present in the serum of diabetic patients (advanced glycation endproducts, AGEs), but also calgranulin, a proinflammatory peptide that derives from the proteolytic

processing of the cytoplasmic protein S100, and amyloid beta-peptide in Alzheimer patients (reviewed by Schmidt et al., 2000). It is expressed on a wide set of cells, including endothelial cells, smooth muscle cells, macrophages/monocytes and neurons, and has been implicated in several pathological processes, such as diabetes, amyloidoses and atherosclerosis. HMGB1 binding on the cell surface itself induces the transcriptional upregulation of RAGE (Li et al., 1998).

The understanding of the signal transduction pathways activated by HMGB1 binding to RAGE is still fragmentary. Extension of neurites requires the small GTPases Cdc42 and Rac, but not the ras-MAP kinase pathway (Huttunen et al., 1999). However, in neural cells, HMGB1 binding to RAGE also activates the Ras-MAP kinase pathway and leads ultimately to the activation of NF-κB, the transcription factor classically linked to inflammatory processes (Huttunen et al., 1999). During tumour invasion the MAP kinases $p38^{MAPK}$, JNK and p42/p44 MAPK have been shown to be activated by RAGE-HMGB1 (Taguchi et al., 2000). HMGB1-mediated migration of smooth muscle cells also results in activation of the MAP kinase pathway and translocation of phosphorylated ERK 1 and 2 into the nucleus, but also involves a $G_{i/o}$ protein (Degryse et al., 2001). How and if these pathways are linked remains an open question; moreover, different pathways may be activated in different cell types. One potentially interesting observation is that cells that respond to extracellular HMGB1 appear to contain very little HMGB1 themselves, and almost none in the nucleus. In support of this observation, it has been shown that *Hmgb1*$^{-/-}$ embryonic fibroblasts respond better to extracellular HMGB1 in cell migration assays than their wild type counterparts (B. Degryse and M.E. Bianchi, unpublished data).

The second role of HMGB1, as an extracellular signal, has been now extensively documented, and is well established. Together, all the data indicate that, as in the nucleus, also outside the cell HMGB1 is a very versatile molecule, involved in several processes: many cellular types can read out the HMGB1 signal, and depending on the target cells very different biological responses are induced (**Fig. 8**).

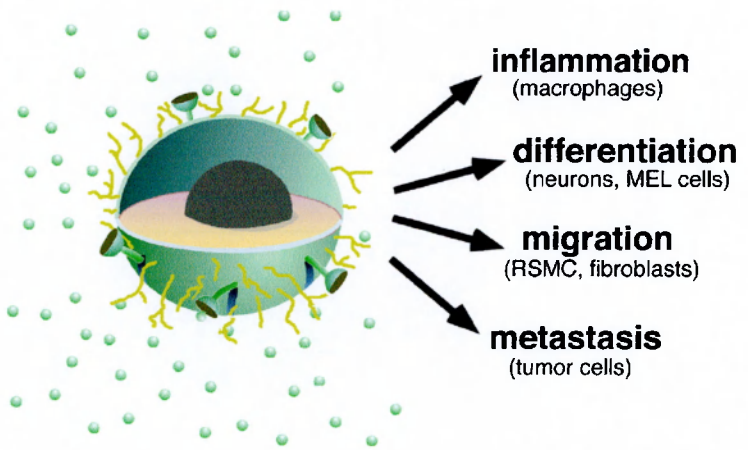

Fig. 8 Different biological responses induced by extracellular HMGB1. Many cells are equipped to recognise HMGB1 in the extracellular milieu. The protein binds with moderate affinity to the proteoglycans covering the surface of every cell (yellow lines). In addition, high affinity receptors like RAGE (green structures) mediate cell-specific responses to HMGB1, triggering different responses, involved in physiological and pathological processes.

1.7 HMGB1: one molecule, many functions

The discovery of the extracellular functions of HMGB1 has opened an entirely new field of investigation and raised several important questions, concerning the mechanisms that regulate its release from the cells, the identity of all its cell surface receptors, and the basis for signal transduction following HMGB1 stimulation. Another important point is understanding how the intracellular and the extracellular roles of HMGB1 are linked to each other, and why a chromatin molecule is employed as an extracellular signal. My thesis work addresses part of this issue, and starting from the analysis of the chromatin binding properties of HMGB1, culminates with the proposal that the passive release of this abundant chromatin protein by dead cells may represent a "danger signal" which triggers inflammation and activates the immune system.

2 MATERIALS AND METHODS

2.1 Cell culture and induction of cell death

HeLa and 3T3 cells and embryonic fibroblasts obtained from wt and *Hmgb1*$^{-/-}$ (Calogero et al., 1999) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS from GIBCO), 100 IU/ml of penicillin and 100 μg/ml streptomycin, in 5% CO_2 humidified atmosphere.

Apoptosis was induced by treating the cells for 16 h with 2 ng/ml human TNF-α and 35 μM cycloheximide (Sigma). Human TNF-α, purified by affinity chromatography, was kindly provided by Flavio Curnis (Dibit, Milan).

Necrosis was induced by treatment for 16 h either with 5 μM ionomycin (Sigma) and 20 μM CCCP (Sigma), or 6 mM deoxyglucose (Sigma) and 10 mM sodium azide, in DMEM serum free. Alternatively, to avoid the presence of chemical poisons in cocultures with bone marrow cells, necrotic cells were obtained by 3 cycles of freeze-thawing in PBS (Phosphate-buffered saline), in liquid nitrogen.

2.2 Immunofluorescence

Cell cultured in LabTek II chambers (Nalgene) were washed first with PBS and then with CSK buffer [10 mM Pipes-KOH, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$ (Fey et al. 1986)]. At this stage, cells were either directly fixed in 4% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature, or subjected to detergent extraction. In the latter case, cells were incubated in CSK buffer

supplemented with 0.15% Triton X-100 or NP-40, 0.5 mM PMSF (Sigma Chemical Co.) and 10 μg/ml leupeptin (Roche) for 1 min at room temperature. After one wash in CSK buffer, the cells were fixed in 4% paraformaldehyde in PBS. After fixation, cells were washed with PBS and incubated for 45 min with blocking solution (3% Bovine Serum Albumin, 0.05% Tween-20 in PBS) containing 0.1% Triton X-100 as permeabilising agent. Primary antibodies were then diluted in blocking solution to the suitable final concentration and incubation was prolonged for 45 minutes at room temperature. After three rinses with PBS containing 0.05% Tween-20 (PB-Tween), cells were incubated with secondary antibodies in blocking solution for 45 minutes, washed three times with PB-Tween, and finally mounted with glass coverslips using Vectashield (Vector Laboratories), containing 1.5 μg/ml DAPI (4',6-diamidino-2-phenylindole) to stain DNA. Cells were analysed on an Axiophot microscope (Carl Zeiss) using Zeiss 40x, 63x and 100x neofluar lenses. Fluorescence photographs were taken on T-Max 400 film (Eastman Kodak), or digital images were acquired using a CCD camera (Hamamatsu Photonics) and HiPic software. The polyclonal rabbit anti-HMGB1 was purchased from BD PharMingen (Torrey Pines, CA), and used diluted 1:1600 in blocking solution. Monoclonal antibody against GFP (working dilution: 1:500), and goat polyclonal antibodies against mouse or rabbit IgG (H+L) conjugated to fluorescein or rhodamine (working dilution: 1:300) were from Boehringer-Roche.

2.3 Differential permeabilisation of cells

HeLa cells were grown to ~ 50% confluence in 6 cm plates and forced to undergo apoptosis as described in paragraph 2.1. After 16 hours, apoptotic cells detached from the plate were collected, centrifuged 5 min at 800 rpm and washed with PBS. A control population of untreated living cells were recovered by trypsinisation and centrifugation.

Both populations were then washed with ice-cold transport buffer (TB buffer), containing 20 mM Hepes, pH 7.3, 110 mM K-acetate, 5 mM Na-acetate, 2 mM Mg-acetate, 1 mM EGTA, 2 mM DTT, and pepstatin, antipain and leupeptin at 1 μg/ml each (Adam et al., 1990). Cells were incubated for 5 min on ice in 50 μl of TB buffer with 0.1% NP-40 or 40 μg/ml digitonin (Sigma Chemical Co.), and centrifuged for 7 min at 700 rpm. Supernatants were recovered and cell remnants were dissolved in 1x SDS-PAGE loading buffer (50 mM Tris pH 6.8, 2% 2-mercaptoethanol, 4% SDS, 12% glycerol, 0.05% bromophenol blue). Concentrated SDS-PAGE loading buffer was added also to supernatants, and samples were analysed by western blot.

In case of necrotic cells, no detergent was added to the cells. The medium from treated and untreated cells was collected and concentrated 50-fold using Amicon Ultrafree-MC filters; the cells were dissolved on the plate in SDS-PAGE loading buffer and analysed by Western blot.

2.4 Detection of proteins by Western blots and Coomassie staining

Samples in SDS-PAGE loading buffer were heated for 5 minutes at 100°C, and applied to SDS polyacrylamide protein gels. The separating gel consisted of polymerised acrylamide-bisacrylamide (ratio 29:1) in 375 mM Tris pH 8.8, 0.1% SDS, at concentration variable from 10% to 13%, depending on the size of the proteins to visualise. The stacking gel consisted of 4% acrylamide-bisacrylamide, 125 mM Tris pH 6.8, 0.1% SDS. The running buffer contained 144 g/l glycine, 30 g/l Tris, 0.5% SDS. The gels were run at 5-15 Volt/cm.

For Western blots, the proteins separated by SDS-PAGE were transferred onto nylon Immobilon P membranes (Millipore) using a tankblot system (Hoefer) in 25 mM Tris pH 7.5, 0.192 M glycine, 20% methanol. Blots were blocked for 1 hour at room

temperature in 5% skim milk /TBST (20 mM Tris, pH 7.5, 137 mM NaCl, 0.1% Tween 20), washed in TBST and incubated for 1 hour at room temperature with anti-HMGB1 antibody (working dilution: 1:3000 in TBST/0.01% BSA). After three additional washes in TBST, filters were further incubated for 1 hour with an anti-rabbit antibody conjugated to HRP (Amersham) (1:5000 in TBST/0.01% BSA). In addition to HMGB1, lactate dehydrogenase (LDH) was visualized by Western blot using a goat polyclonal primary antibody (Chemicon, 1:500 in 5% skim milk /TBST), and a rabbit anti-goat IgG-HRP conjugated antibody (Santa Cruz Biotechnology). The polyclonal antibody R10, a kind gift from B.M. Turner (University of Birmingham Medical School), was used to detected specifically the acetylated form of histone H4. After incubation with secondary antibodies, blots were washed with TBST and developed using the ECL system (Amersham).

Coomassie staining was used to detect histones. After electophoresis, gels or part of the gels containing histones were incubated for 45 minutes in staining solution, containing 45% EtOH, 8% acetic acid, 5 g/l Coomassie blue, and then in destain solution, containing 23% EtOH, 8% acetic acid, until proteins were clearly visible. Gels were finally dried on 3MM paper with a gel drier.

2.5 Two dimensional gel electrophoresis

Total extracts from ~ 10 million living and apoptotic HeLa cells were obtained by 3 cycles of freeze-thawing in 80 µl of Extraction buffer (50 mM PIPES pH 7, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, and pepstatin, antipain and leupeptin at 1 µg/ml each). Extracts were centrifuged for 5 min at 13000 rpm to pellet membranes and 40 µl of the supernatants (total amount of proteins: about 150 µg) were added to 320 µl of rehydration buffer, containing 8 M urea, 2% CHAPS, 20 mM dithioerythritol (DTE),

0.8% IPG buffer (carrier ampholytes, pH 3-10, Pharmacia Biotech). Samples were applied onto ceramic strip holders (Pharmacia Biotech), connecting two electrodes, in contact with 18 cm polyacrylamide gel strips (pH range: 3-10, Pharmacia Biotech). Isoelectrophocusing (IEF) was performed on IPGphor (Pharmacia Biotech) with the following protocol:

-rehydration: 30 min at 20°C

IEF: 18°C

-S1: step-n-hold 30 V, 10.0 hours

-S2: step-n-hold 200 V, 1.5 hours

-S3: gradient 3500 V, 2.5 hours

-S4: step-n-hold 3500 V, 2.0 hours

-S5: gradient 8000 V, 1.5 hours

-S6: step-n-hold 8000 V, 6.0 hours

The IEF was stopped after 75000- 90000 V/h. Second dimension electrophoresis runs were performed using a Protean II apparatus (Bio-Rad), connected to a cooled recirculating bath. After IEF, strips were soaked first for 10 min at room temperature in Equilibration buffer (EB: 6 M urea, 3% SDS, 375 mM Tris pH 8.6, 30% glycerol) containing 2% DTE, then for further 10 min in EB containing 3% iodoacetamide (IAA) and traces of bromophenol blue (BBP). Strips were then applied onto 10% polyacrylamide gels in 375 mM Tris pH 8.8. The running buffer contained 144 g/l glycine, 30 g/l Tris base, 0.5% SDS. Gels were run in the same apparatus at 90 V for about 13 hours, until BBP was near the bottom of the gel. Proteins were then transferred onto nitrocellulose membranes (Schleicher & Schuell) using a tank blot system (Hoefer) in 25 mM Tris pH 7.5, 0.192 M glycine, 20% methanol. Western blots were performed as described in the previous paragraph.

2.6 *E. coli* strains

DH5α: Strain with genotype *supE44 DlacU169 (F80 lacZDM15) hsdR17 recA1 end A1 gyrA96 thi-1 relA1.* A recombinant-deficient suppressing strain used for plating and growth of plasmids.

XL1-Blue: Strain with genotype *supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻ F'(proAB⁺lacIᑫlacZDM15*Tn*10(tetʳ)).* A recombination-deficient strain that will support the growth of vectors carrying some amber mutations. Transfected DNA is modified but not restricted. The F' in this strain allows blue/white screening on X-GAL and permits bacteriophage M13 superinfection.

HB101: *supE44 hsdS20($r_B^-m_B^-$) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1.* A suppressing strain commonly used for large-scale production of plasmids. It is an *E. coli* K12 X *E. coli* B hybrid that is highly transformable.

BL21(-): Strain with genotype *hsdS gal (λ clts857ind 1 Sam7 nin5 lacUV5-T7 gene1).* A strain used to express at high levels genes under the control of T7 promoter. It lacks the plasmid pLysE, coding for T7 phage lysozyme which inhibits the RNA polymerase basal activity.

E. coli bacteria were generally grown in LB (Luria-Bertani) Medium supplemented with ampicillin at a concentration of 100 mg/ml or kanamycin at a concentration of 25 mg/ml as selective agents.

LB (per litre):

bacto-tryptone	10g
bacto-yeast extract	5g
NaCl	10g

To express recombinant HMGB1, bacteria were grown in M9 minimal medium:

M9 (per litre):		5 X M9 salts (per litre):	
5 X M9 salts	200 ml	$Na_2HPO_4 \cdot 7H_2O$	64 g
1 M $MgSO_4$	2 ml	KH_2PO_4	15 g
1 M $CaCl_2$	0.1%	NaCl	2.5 g
		NH_4Cl	5 g

2.7 Protocol of plasmid DNA extraction

Plasmid DNA was prepared using two classical techniques of plasmid extraction. The first was used to prepare plasmid DNA from 3 ml of many different cultures (1 to 24) of plasmid-containing bacteria (Maniatis, 1989). The second one was used to isolate large quantities of plasmid DNA, using anionic exchange cartridges produced by QIAGEN. Bacterial cells were lysed under alkaline conditions and the crude lysates were cleared by centrifugation. The cleared lysate was then loaded onto the anion-exchange tip where plasmid DNA selectively binds under appropriate low-salt and pH conditions. RNA, proteins, metabolites, and other low-molecular-weight impurities were removed by a medium-salt wash, and plasmid DNA was then eluted in high-salt buffer. The DNA was concentrated and desalted by isopropanol precipitation and collected by centrifugation.

2.8 Plasmid and constructs

The plasmid pEGFP-HMGB1 contains the open reading frame of HMGB1 fused at the 3' end with the coding region of the Enhanced Green Fluorescent Protein

(Enhanced GFP). It was generated by inserting a 680 bp fragment of rat HMGB1 cDNA (35bp upstream the ATG until the stop codon) into the pEGFP-N1 vector (Clontech) using the EcoRI and SacII restriction sites. Both restriction sites were inserted at the ends of the cDNA by PCR, using the following oligonucleotides:

*Eco*RI

HMGB1-EcoRI for 5' - AGGAATTCCGGTGCCTCGCGGAG - 3'

Sac II

3' HMGB1-GFP rev 5' - ACCCCGCGGTTCATCATCATCATC - 3'

The plasmids pEGFP-H1c (a gift from A. Gunjan), pEGFP-HMGN2 (a gift from R. Hock and M. Bustin) and pEGFP-NF1 (a gift from N. Bhattacharyya) encode GFP fusions to the chromatin proteins histone H1c and HMGN2, and to the general transcription factor NF1.

The plasmid pEF-flag-mICAD (a gift from S. Nagata) is a mammalian expression vector of mouse ICAD-L, tagged at the N-terminus with the Flag epitope.

The pSGD9 construct was kindly provided by V. Zappavigna, and contains the human *HOXD9* gene cloned in the SV40 early-promoter-based mammalian expression vector pSG5 (Zappavigna et al., 1996). The pT81HCR reporter construct contains a single copy of the HOXD9 100 bp autoregulatory element HCR, cloned into the polylinker of the pT81luc luciferase reporter vector (Zappavigna et al., 1996). PRLnull carries the *Renilla* luciferase coding region with no minimal promoter, and was used as internal control of the transfections.

The plasmid pT7-7-rHMG1cm, encoding the full-length HMGB1 protein, was a kind gift of Prof. J.O. Thomas (Cambridge).

2.9 Transient and stable transfections

HeLa cells were transfected by calcium phosphate co-precipitation. To express HMGB1-GFP, 200,000 cells were plated in 6 cm dishes and were transiently transfected with 8 μg of pEGFP-HMGB1. Cells were observed 24-48 hours after transfection. The average amount of HMGB1-GFP in the cell population was between 1 and 3% of HMGB1 (as measured by immunoblotting with anti-HMGB1 antibodies). At the single cell level, the amount of HMGB1-GFP varied at most tenfold between different cells; care was always taken to use for analysis only cells with a moderate fluorescence level.

In the HOXD9 transfection assay, 200,000 cells were transfected with 1.5 μg of reporter plasmid (pTHCR), 1 μg of pSGD9, increasing amounts (0-2 μg) of constructs expressing HMGB1 or HMGB1-GFP (pHMGB1 or pEGFP-HMGB1) and 300 ng of pRLnull as an internal control. Transfection was carried out in triplicate batches. Forty eight hours after transfection, cells were harvested and luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega) and Lumino luminometer (Stratec).

To generate stable transfectants overexpressing ICAD, ~ 600,000 HeLa cells plated in 10 cm dishes were co-transfected by calcium phosphate co-precipitation with 20 μg of pEF-flag-mICAD and 4 μg of the plasmid pcDNA3 carrying neomycin resistance. Seventy two hours after transfection cells were diluted 1:10 and neomycin (Roche) was added to the medium to 0.9 mg/ml final concentration. After ten days of selection, resistant transformants were picked picked with sterile tips, trypsinised, grown in 10-cm dishes and analysed for ICAD expression by Western blot, with anti-Flag monoclonal antibody (M2, Kodak).

2.10 Detection of DNA fragmentation inhibition in cells overexpressing ICAD

To check for the inhibition of DNA degradation, cells from three different clones forced to undergo apoptosis were stained with TUNEL (Apoptosis detection system, Promega), and their chromosomal DNA was extracted and analyzed by electrophoresis on agarose gels.

TUNEL staining measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'OH DNA ends using the enzyme terminal deoxynucleotidyl tranferase (TdT), which forms a polymeric tail (TUNEL assay: TdT-mediated dUTP Nick-End Labelling). Wt and stable transfected cells treated with TNF-α and cycloheximide were permeabilised as described in paragraph 4.2, fixed with 4% methanol-free formaldehyde, incubated for 10 min with equilibration buffer (200 mM potassium cacodylate pH 6.6, 25 mM Tris-HCl pH 6.6, 0.2 mM DTT, 0.25 mg/ml BSA, and 2.5 mM cobalt chloride) and then incubated at 37°C for 1 hour with 50 µl of a solution containing 45 µl of equilibration buffer, 5 µl of nucleotide mix (50 µM fluorescein-12-dUTP, 100 µM dATP, 10 mM Tris-HCl pH 7.6, and 1 mM EDTA), and 25 units of TdT enzyme. Cells were then washed three times with 2X SSC and once with PBS. Slides were finally mounted with Vectashield containing 1.5 µg/ml DAPI, and analysed at the fluorescence microscope, using a FITC filter.

To analyse genomic DNA by gel electrophoresis, ~ 500,000 apoptotic cells, from three different clones overexpressing ICAD and a control population of wt HeLa cells, were recovered, washed with PBS, resuspended in 350 µl Lysis buffer (100 mM Tris pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) containing 500 µg/ml proteinase K and incubated for 4 hours at 56°C. Proteins were removed by double extraction with phenol:cloroform:isoamyl alcohol (25:24:1). DNA was precipitated with 2.5 vol of

100% EtOH and 300 mM Na-acetate pH 5.3, washed with 70% EtOH, resuspended in 30 µl of water, and analysed by electrophoresis on a 1.5% agarose gel.

2.11 *In vivo* microscopy and photobleaching analysis

Living cells expressing HMGB1-GFP were imaged on an Axiovert 135 M microscope (Zeiss), by differential interference contrast (DIC) and in fluorescence imaging.

Photobleaching experiments were performed on a Leica TCS-SP confocal microscope using the 488 nm laser line of an Ar laser (20 mW nominal output, beam width at specimen 0.2 µm, detection 500–575 nm). All experiments were done at 37°C. For Fluorescence Recovery After Photobleaching (FRAP) experiments, five single scans were acquired, followed by a single bleach pulse of 200–500 ms using a spot 1 µm in radius without scanning. Single section images were then collected at 1.6 s intervals. For imaging, the laser power was attenuated to 1% of the bleach intensity. For Fluorescence Loss In Photobleaching (FLIP) experiments, cells were repeatedly imaged and bleached at intervals of 6 s. Bleaching and imaging settings were identical to the ones used in FRAP.

FRAP and FLIP curves were generated from background subtracted images. For FRAP, the total cellular fluorescence was determined for each image and compared with the initial total fluorescence to determine the amount of signal lost during the bleach pulse and during imaging. Typically 10% of total fluorescence was lost during the bleach pulse and less than 5% of fluorescence was lost during the entire imaging phase. The fluorescence signal measured in a region of interest normalized to the

change in total fluorescence was determined as

$$I_{rel} = \frac{T_0 \; I_t}{T_t \; I_0}$$

where T_0 is total cellular intensity during prebleach, T_t the total cellular intensity at time point t, I_0 the average intensity in the region of interest during pre-bleach, I_t the average intensity in the region of interest in time point t. This expression accounts for the small loss in total intensity caused by the bleach itself and yields a more accurate estimate of the immobile fraction. The relative intensity in the bleached region was plotted as function of the recovery time. For FLIP, the relative intensity of fluorescence (I_t / I_0) was measured in different areas of the cell, distant from the bleached region, and the mean values were plotted as a function of time. Recovery and loss of fluorescence curves for each protein were generated on the basis of several measurements from at least ten different cells, showing moderate fluorescence levels.

2.12 Binding of recombinant HMGB1 to chromatin

Hmgb1$^{-/-}$ fibroblasts were treated with 2 ng/ml hTNF-α and 35 μM cycloheximide. After 16 h, apoptotic cells were recovered by gentle flushing of the dish. Ten million apoptotic *Hmgb1*$^{-/-}$ fibroblasts and a control population of non-apoptotic ones were resuspended in 50 μl PBS containing 0.32 M sucrose, 0.5% NP-40 and 1 μM bacterially produced HMGB1, either fluorescently labelled with Cy5 (Cy5 mono-reactive Dye Pack, Pharmacia) or unlabelled. Average labelling was 2.3 Cy5 molecules per HMGB1 molecule. After 30 minutes at room temperature, apoptotic and non-apoptotic cells incubated with Cy5-HMGB1 were mixed and mounted on slides using

Vectashield containing 1.5 μg/ml DAPI, and observed on an Axiophot microscope (Carl Zeiss) with rhodamine filter. The two pools of cells incubated with unlabelled HMGB1 were layered onto discontinuous gradients, formed by 5 ml of 1.16 M sucrose in PBS and a 6 ml cushion of 2 M sucrose in PBS. The gradients were centrifuged at 30,000 x g for 90 minutes in a SW27 Beckman rotor. Apoptotic and non-apoptotic chromatin free from membrane debris was recovered from the bottom of the tubes, dissolved in SDS-PAGE loading buffer and electrophoresed through a 12% SDS-PA gel. The amount of recombinant HMGB1 bound to apoptotic and non-apoptotic chromatin was detected by Western blot. Aliquots of apoptotic and non-apoptotic chromatin were also probed with various antibodies: anti-acetyl-histone H4 (R10, a gift from B. Turner), anti-acetyl-Histone H3 (Lys 9, Biolabs) and anti-acetyl-lysine antibody (Biolabs) to check the acetylation status of core histones.

2.13 Bone marrow extraction and stimulation by necrotic and apoptotic cells

Thighbones from the hind legs of female C56Bl6 mice were recovered and cut at both ends. Bone marrow cells were obtained by flushing with 1.5 ml of PBS using a 1 ml syringe. After centrifugation at 1200 rpm for 5 min, cells were diluted to 5 x 10^6 cells/ml in Optimem (Gibco), dispensed in 96-well plates (120 μl per well) and left to attach for three hours before stimulation.

Necrotic wt and *Hmgb1*$^{-/-}$ fibroblasts were obtained by 3 cycles of freeze-thawing in liquid nitrogen. Living cells were trypsinised and resuspended in PBS to a final concentration of 25.000 cells/μl prior to lysis. Apoptotic cells, obtained by TNF-α/cycloheximide treatment, detached from the plate, were recovered and washed twice in PBS. Necrotic and apoptotic cells were added to increasing final concentrations into

the wells containing bone marrow cells and incubated at 37°C for 18 hours. TNF-α released in the supernatant was assayed by ELISA (Quantikine M, R&D Systems).

2.14 Induction of liver necrosis by acetaminophen overdose

One day old mice (weighing 1.1± 0.1 grams) were injected intraperitoneally with 20 µl of PBS containing 320 µg of acetaminophen (Sigma), and 320 µg of antibodies (either anti-HMGB1, or rabbit IgG form Sigma) where indicated. After 9 hours mice were decapitated, blood was collected, and liver was taken. Blood samples were allowed to clot overnight at 4° C, sera were recovered and analyzed for alanine transaminase (ALT) activity with the GP-Transaminase kit (Sigma). The recruitment of inflammatory cells was quantified by assessing the liver myeloperoxidase (MPO) content. Liver samples were homogenized in buffer B (43.2 mM KH_2HPO_4, 6.5 mM Na_2HPO_4, 10 mM EDTA, 0.5% hexadecyltrimethylammonium, pH 6.0) (2 ml of buffer B per 100 mg of tissue). After heating for 2 hours at 60° C, 66 µl of homogenised liver were reacted with 133 µl of 3,3',3,5'-tetramethylbenzidine (Sigma) for 8 min. Samples were then centrifuged for 3 minutes at 13,000 rpm, and the optical density of the supernatant was determined at 655 nm (Kato et al., 2000)

Statistical analysis was performed with the non-parametric Mann-Whitney test on MPO/ALT ratios, using Prism software.

2.15 Purification of recombinant HMGB1 (by Tiziana Bonaldi)

A colony of BL21(-) freshly transformed with the plasmid pT7-7-rHMG1cm was used to inoculate an overnight culture grown in M9 medium complemented with 20 g/l cas-aminoacids, 0.5% glycerol, 5 g/l yeast extract, 0.4% glucose and 100 µg/ml

chloramphenicol. The overnight culture (300 ml) was then used to inoculate a 3 l culture in an ADI 7 l autoclavable bioreactor (Applikon). At an optical density OD_{595} of 0.7, IPTG was added to the culture to a final concentration of 0.5 mM. Shaking was reduced to 150-200 rpm and temperature to 23°C. Growth was continued for another 16 hours. Cells were centrifuged in GSA rotor 5 Kpm for 10 min at 4°C, resuspended in 120 ml of buffer L2 (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 1 mM DTT, 0.5 mM PMSF) and sonicated with a large piston 6 times for 1 minute. NaCl was added to a final concentration of 0.5 M. After 5 min on ice, the suspension was centrifuged at 10 Kpm 10 min at 4°C. Solid $(NH_4)_2SO_4$ (3.9 g) was added for every 10 ml (final 60% solubility). After 30 min on ice, the solution was centrifuged at 10 Kpm for 10 min in a SS-34 rotor at 4°C. The supernatant was loaded on a Phenyl-Sepharose Pharmacia column connected to a FPLC system; the protein was eluted with stepwise decreasing elution (60, 50, 40, 30%) in Buffer B (20 mM HEPES pH 7.9, 0.5 mM DTT, 0.2 mM PMSF, 0.2 mM EDTA pH 8.0, 60% $(NH_4)_2SO_4$). The positive fractions were collected, pooled and dialysed overnight in 500 ml Buffer A' (50 mM Hepes pH 7.9, 0.5 mM DTT, 0.2 mM PMSF, 20 mM NaCl). The dialysed sample was loaded on a Hi-trap SP column (Pharmacia) and eluted with an increasing ionic-strength buffer (20mM-1M NaCl, 20 mM HEPES pH 7.9, 0.5 mM DTT, 0.2 mM PMSF, 0.2 mM EDTA). The sample was concentrated with Centricon-10 cartridges (Amicon) and desalted with PD-10 columns (Pharmacia), and stored in phosphate buffer pH 7.5, containing 0.5 mM DTT, 0.2 mM PMSF, 0.2 mM EDTA.

3 RESULTS

3.1 Subcellular localisation of HMGB1 in living cells

Previous studies have shown that HMGB1 binds with high affinity to *in vitro* reconstituted nucleosomes but, contrary to other chromatin binding proteins, is only loosely bound to chromatin, and rapidly leaks out into the medium when cellular membranes are permeabilised (Falciola et al., 1994). This observation was interesting but puzzling: I therefore decided to gain insight into this issue and better characterize the chromatin binding properties of HMGB1 within the cell.

In order to analyse the behaviour of the protein in living, unperturbed cells, I employed non-invasive techniques, based on the Green Fluorescent Protein (GFP).

3.1.1 The GFP technology

The use of the GFP in cellular and molecular biology has given an extraordinary contribution towards the understanding of how events occur inside of living cells.
GFP is a protein of the jellyfish *Aequorea victoria*, and is responsible for the green lining along the margins of the jellyfish's bell. GFP is unique among light-emitting proteins in that it does not require the presence of any cofactors or substrates for the generation of its green light. In the jellyfish, GFP is activated in a Ca-dependent manner by another bioluminescent protein, aquorin, which transfers energy indirectly to GFP to trigger the release of green light (Prasher, 1995). This energy transfer can be mimicked experimentally by simple exposure of GFP to standard long-wave ultraviolet (UV) light.

The crystal structure of GFP has been solved and shown to be a remarkable barrel-like arrangement (Ormö et al. 1996): eleven β-sheets surround a central α-helix, containing the fluorescent centre, which is formed by a hexapeptide (**Fig. 9**). The chromophore itself is formed by a Ser65-Tyr66-Gly67 tripeptide, which forms in an as-yet uncharacterised cyclization process in the presence of oxygen (Heim et al., 1994). Mutational analysis of this and other regions of the protein have produced "improved" GFP isoforms, brighter and more resistant to photobleaching (Heim and Tsien, 1996).

GFP is already an invaluable tool to monitor protein localisation in living cells and to visualize dynamic cellular events. A fusion between any cloned gene of interest and GFP can be produced by standard subcloning techniques and may be introduced into the organism of interest by transient or stable expression. The fate of the resulting protein inside the living cells can be followed by conventional fluorescence microscopy. Importantly, detection does not require fixation or permeabilisation of cells; therefore, compared with immunocytochemistry techniques using fixed cells, the likelihood of artifacts is reduced. On the other hand, the addition of relatively large GFP-tag of 27 KDa can affect the functionality of the fused protein of interest. It is therefore necessary that each fusion protein be carefully tested for its functionality *in vivo*.

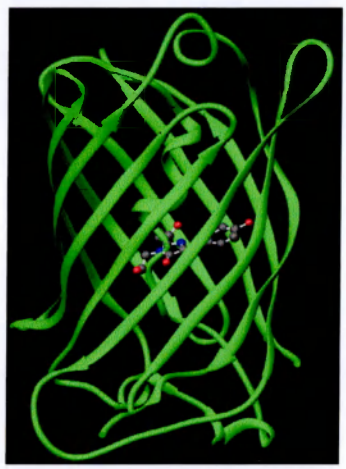

Fig. 9 Three-dimensional structure of GFP. Ribbon diagram of the Green Fluorescent Protein (GFP) drawn from the wild-type crystal structure. The buried chromophore, which is responsible for GFP's luminescence, is shown in full atomic detail.

3.1.2 Generation and expression of HMGB1-GFP in HeLa cells

I generated a chimeric protein in which the Green Fluorescent Protein is fused to the C-terminus of HMGB1 (**Fig. 10**).

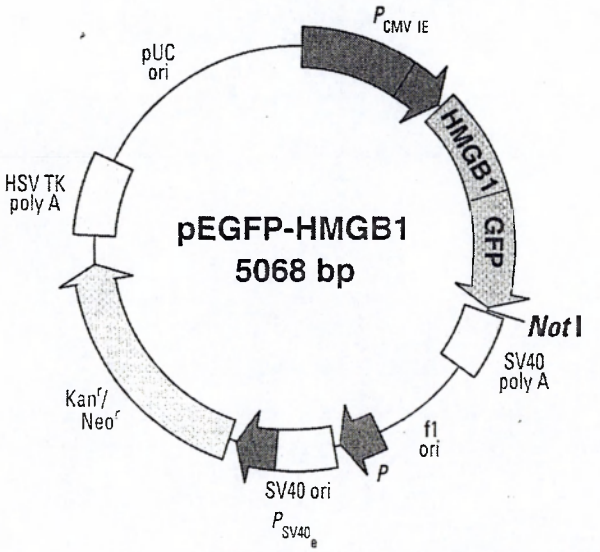

Fig. 10 Plasmid encoding GFP-HMGB1. The HMGB1 coding region is fused to the GFP open reading frame, in the mammalian expression vector pEGFP-N1 (Clontech). Cell transfected with the plasmid express HMGB1 fluorescently labelled by the c-terminal GFP.

The coding sequence of HMGB1 was inserted upstream the 5' end of the GFP open reading frame. We employed for our studies the enhanced version of GFP, which incorporates silent base mutations corresponding to human codon-usage preferences and allows a higher expression in mammalian systems. Enhanced GFP (EGFP) also includes chromophore mutations which increase its fluorescent intensity and make it less susceptible to photobleaching.

In order to study the subcellular localisation of HMGB1 in living cells, HeLa cells were transfected with pEGFP-HMGB1 and analysed on an inverted microscope. In interphase, HMGB1-GFP was entirely localised in the nucleus of the cells, showing a uniform, finely punctate distribution in the whole nucleoplasm, with a less intense

staining on nucleoli (**Fig. 11A**). An identical pattern of staining is obtained by detecting endogenous HMGB1 in fixed cells by indirect immunofluorescence (**Fig. 11B**).

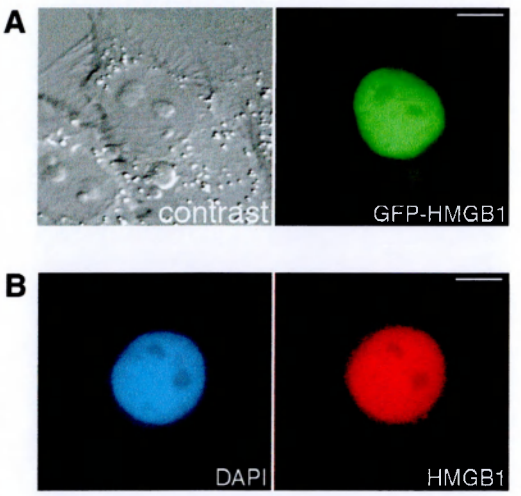

Fig. 11 Subcellular localisation of HMGB1 in interphase cells. Scale bars, 7.5 μm.
A. Living HeLa cells expressing HMGB1-GFP were imaged by differential interference contrast microscopy and in green fluorescence. In living cells, HMGB1-GFP is completely localised within the nucleus, showing a lower concentration in the nucleoli.
B. Hela cells were fixed with PFA and stained for HMGB1 and DNA. A distribution identical to that observed in living cells is obtained also in fixed cells by immunodetecting the endogenous protein.

Cells undergoing mitosis, instead, showed a low diffuse cytoplasmic fluorescence, but also a distinct association of HMGB1-GFP to condensed chromosomes, lasting throughout M phase (**Fig. 12A**). This observation was unexpected since no HMGB1 bound to mitotic chromosomes can be detected in fixed cells by

immunofluorescence (**Fig. 12B**), and the protein appears totally displaced from condensed chromatin.

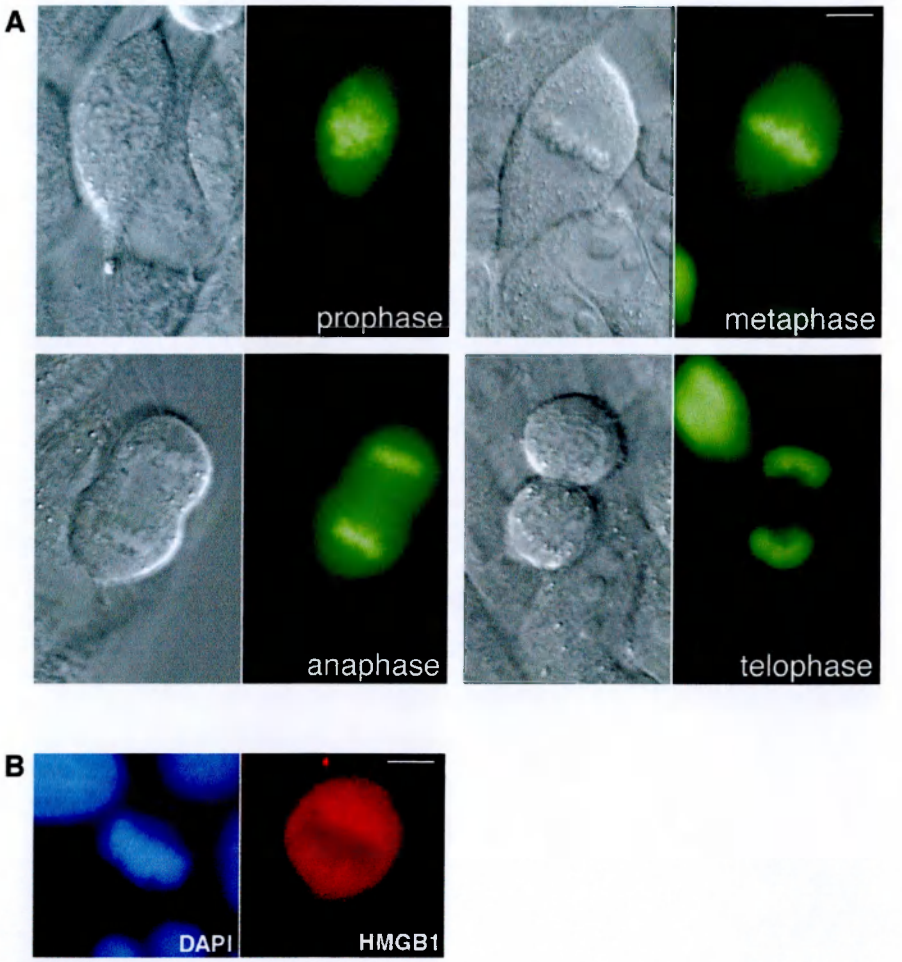

Fig. 12 Subcellular localisation of HMGB1 in mitotic cells. Scale bars, 6 μm.

A. In living cells, during mitosis HMGB1-GFP is distributed in the whole cell but is highly concentrated onto condensed chromosomes

B. An opposite distribution is observed in fixed cells, by immunodetecting the endogenous HMGB1: the protein is displaced from chromosomes and accumulates in the cytoplasm.

I investigated the cause of the discrepancy between data obtained in living and fixed cells and found that fixation abolishes the association of HMGB1 with chromosomes. When HMGB1-GFP expressing cells are fixed, with paraformaldehyde, acetone, and/or acetic acid, an immediate loss of fluorescence from the chromosomes is observed. This is not due to specific fluorescence quenching within the chromosomes, as indirect immunofluorescence with anti-GFP antibodies confirmed the loss of the association of the fusion protein to the chromosomes (**Fig. 13**).

We concluded that in living cells HMGB1 is bound to the condensed chromatin, but this association is not stable and is rapidly lost upon cell manipulation.

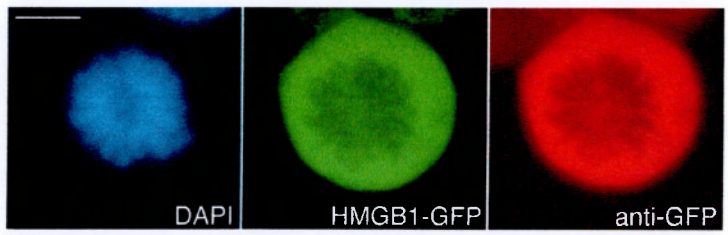

Fig. 13 Effect of fixation on the binding of HMGB1 to chromatin. HeLa cells expressing HMGB1-GFP were fixed with 4% PFA. Indirect immunofluorescence was performed using an anti-GFP antibody and a TRITC-coniugated secondary antibody, and DNA was stained with DAPI. Following fixation, HMGB1-GFP is detached from chromosomes. Scale bar, 6 μm.

3.2 Functionality of HMGB1-GFP

In order to check the functionality of the chimeric protein, I compared HMGB1-GFP with the unmodified HMGB1 in its ability to enhance the expression of a HOXD9-responsive reporter gene in transfection assays. HMGB1 is one of the co-factors cooperating with HOX proteins in the recognition of their specific target sequence. HMGB1 and HOXD9 establish protein-protein contacts via their HMG-boxes and homeodomain, respectively, and in transient transfection assays, co-expression of HMGB1 and HOXD9 enhances up to 4-fold the transcriptional activation of a reporter gene carrying HOXD9 target sequence (HCR) (Zappavigna et al., 1996). In this assay, I used as a reporter plasmid, pTHCR, where the HCR element is fused to the herpes simplex virus thymidine kinase (TK) promoter, upstream the luciferase coding region. HeLa cells were transiently co-transfected with fixed amounts of pTHCR and the HOXD9 expression construct pSGD9, and increasing amounts of either pEGFP-HMGB1 or pHMGB1, which contains the whole *Hmgb1* gene under the control of its own promoter (See Materials and Methods). pEGFP-HMGB1 was able to stimulate the HOXD9-mediated transcription in a way comparable to pHMGB1 (**Fig. 14**), showing that the presence of the fused GFP does not impair the transactivational activity of HMGB1

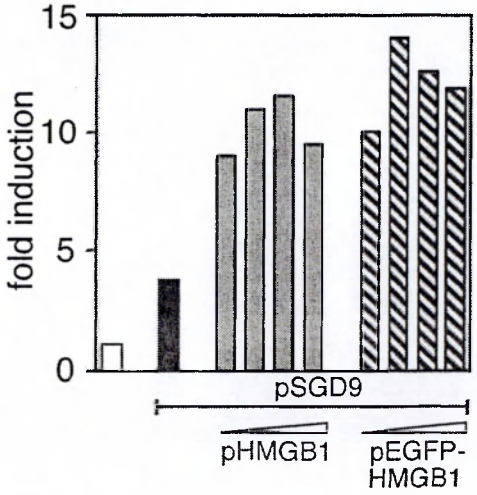

Fig. 14 The fusion to GFP does not impair the transactivational activity of HMGB1. HeLa cells were transfected with fixed amounts of the reporter plasmid pTHCR and pSGD9, and increasing amounts of either pHMGB1 or pEGFP-HMGB1. Both HMGB1 and HMGB1-GFP enhance HOXD9 transcriptional activity in transient co-transfection assays.

3.3 Binding of HMGB1-GFP to chromatin in living cells

I then verified whether HMGB1-GFP behaves like endogenous HMGB1 upon cell permeabilisation. HMGB1-GFP expressing cells, observed on an inverted microscope, were treated with 0.15% Triton X-100 or NP-40, and sequential images were collected (**Fig. 15**). Already 4 seconds after the addition of detergents, HMGB1-GFP started to diffuse out of the cells, both in mitosis and in interphase, and after 80 seconds cells were completely devoid of fluorescent protein. Thus, HMGB1-GFP, like endogenous HMGB1, is only loosely associated with chromatin in living cells, and if membranes are permeabilised, all the protein leaks out of the cell.

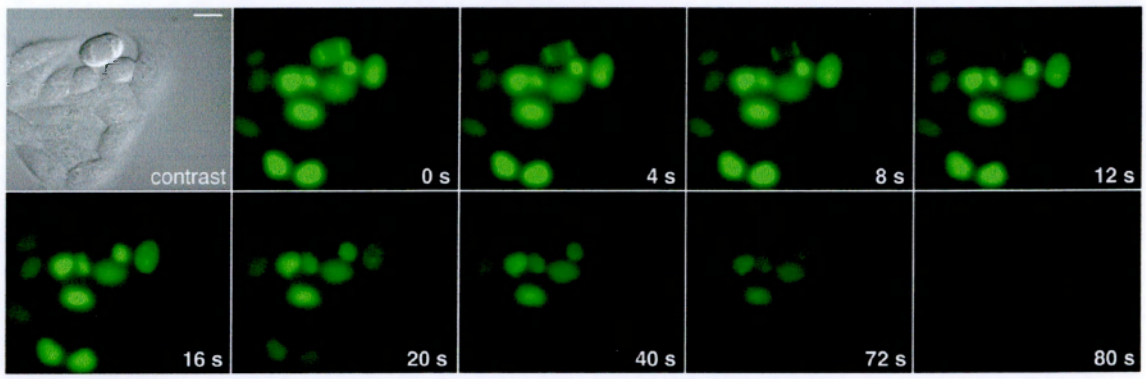

Fig. 15 Leakage of HMGB1-GFP from permeabilised cells. HeLa cells expressing HMGB1-GFP were permeabilised with NP-40 directly on the microscope, and sequential images were collected before and after detergent addition. Less then 1.5 min are sufficient to lose the entire complement of HMGB1 upon permeabilisation. Scale bar, 12 μm.

3.4 HMGB1 is retained in permeabilised apoptotic cells

Even though most HMGB1-GFP transfected HeLa cells lost their fluorescence a few seconds after NP-40 addition, some cells retained a bright fluorescence even after minutes (**Fig. 16**). From the blebbed membranes and the characteristically fragmented appearance of their nuclei, the fluorescence-retaining cells appeared to be apoptotic.

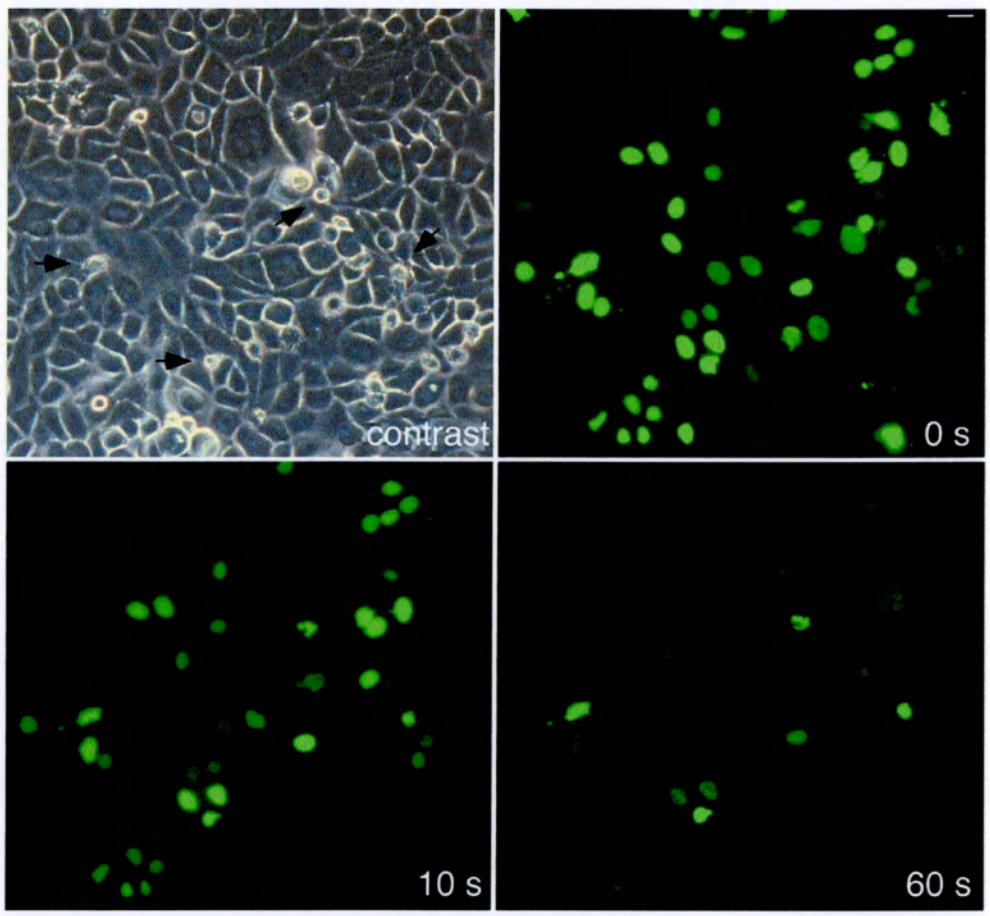

Fig. 16 Retention of HMGB1 by some permeabilised cells. Not all the cells released HMGB1 after detergent treatment. Even after minutes, some cells maintained their green fluorescent signal. Observed at bright field, these cells (arrows) looked morphologically different from the other cells, and appeared to be apoptotic. Scale bar, 15 μm.

To test directly whether HMGB1 was specifically retained by apoptotic cells, I forced HeLa cells to undergo apoptosis by treatment with Tumor Necrosis Factor-α (TNF-α) and cycloheximide, and immunostained them for their endogenous, unmodified HMGB1. Cells were treated with NP-40 before fixation, and HMGB1 was detected using an anti-HMGB1 antibody. As expected, control non-apoptotic cells appeared devoid of HMGB1, which had been released from the nucleus into the medium (**Fig. 17A**). On the contrary, apoptotic cells, recognizable by their condensed and fragmented chromatin, showed an intense staining for HMGB1, suggesting that all the protein had been retained within the nucleus even after membrane solubilisation (**Fig. 17B**).

The retention of HMGB1 by permeabilised apoptotic cells was also revealed by Western blotting. After NP-40 treatment, soluble proteins released from the nucleus were separated from histones and chromatin-associated proteins by centrifugation. In control cells, HMGB1 leaked completely into the supernatant (**Fig. 17A**); in contrast, with apoptotic cells, the majority of the protein was found in the pellet, associated with histones, suggesting that in apoptosis the binding of HMGB1 to chromatin was more stable (**Fig. 17B**).

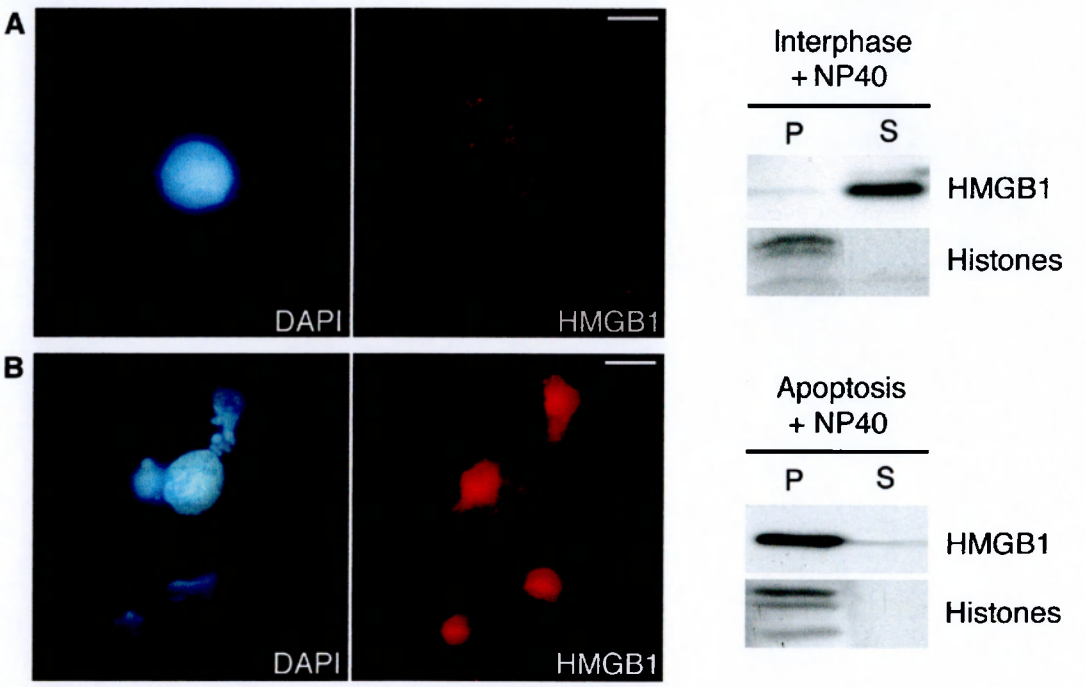

Fig. 17 Chromatin association of HMGB1 in permeabilised living and apoptotic HeLa cells. The medium bathing the cells (S) was electrophoresed alongside with the cells dissolved in loading buffer (P). Histones were visualized by Coomassie staining, HMGB1 by immunoblotting. Scale bars, 7.5 μm. **A.** Interphase cells were permeabilised with 0.1% NP-40, fixed with PFA, and stained with DAPI and anti-HMGB1 antibodies. The nucleus was devoid of HMGB1 and most of the protein was found in the medium. **B.** HMGB1 was firmly bound to the chromatin of apoptotic cells (recognizable by the their fragmented nuclei), even after NP-40 permeabilisation: the apoptotic nuclei showed an intense staining, and most HMGB1 was found associated to the hisones.

This behaviour appeared to be a specific feature of HMGB1, and not a general phenomenon affecting all the proteins in apoptosis. PCNA (Proliferating Cells Nuclear Antigens), for example, a DNA binding protein involved in DNA repair, is also sensitive to detergents, unless DNA damage is induced; permeabilised apoptotic cells however released the protein not unlike living cells (**Fig. 18 A**). Identical results were

observed for HMGB1 also using different apoptotic stimuli, such as etoposide or H_2O_2, and even in cells spontaneously undergoing apoptosis in unperturbed cultures, HMGB1 was not sensitive to permeabilisation. In the same conditions, HMGB1-GFP behaved like the endogenous protein, contrary to control unfused GFP, which was always released upon NP-40 treatment (**Fig.18 B**).

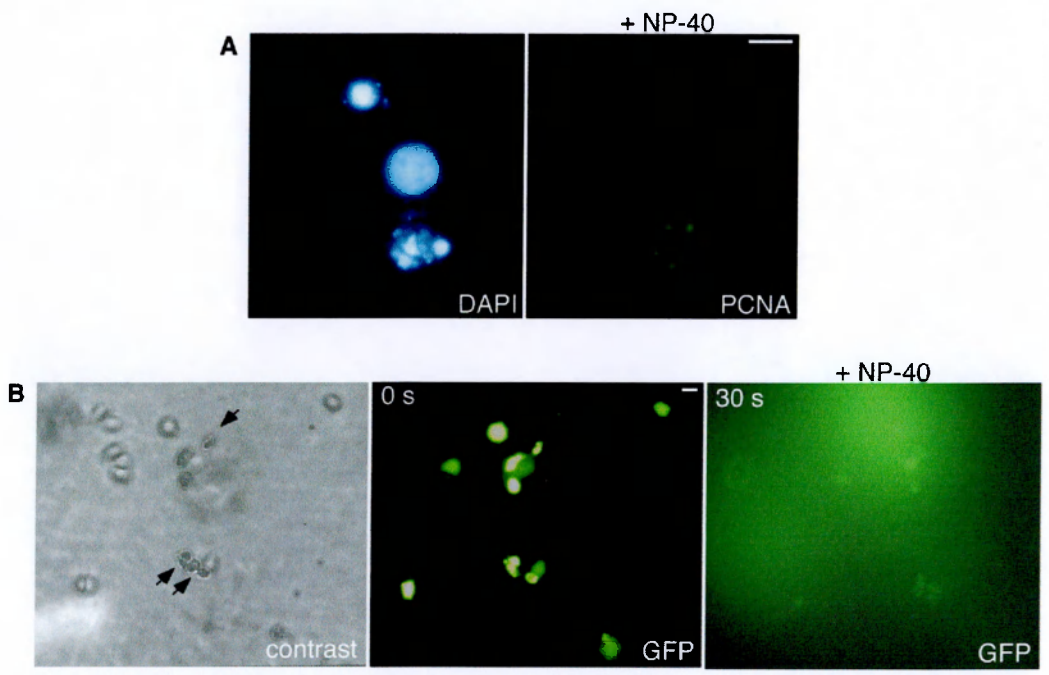

Fig. 18 Apoptotic cells retain specifically HMGB1. Scale bars, 10 μm.

A. Immunofluorescence on TNF-α-treated HeLa cells. Cells were permeabilised with NP-40, fixed and immunostained for DNA and PCNA. Apoptotic cells released PCNA and appeared devoid of the protein as well as non-apoptotic cells.

B. Cells expressing unfused GFP were permeabilised with NP-40 while imaged on the microscope. Already 30 seconds after detergent addition all the cells lost GFP, including cells spontaneously undergone apoptosis (arrows).

The permeabilisation of apoptotic cells mimics what happens in the latest stage of the apoptotic process, when cells undergo partial autolysis and lose their membrane integrity even in the absence of detergents. I checked the behaviour of HMGB1 in this condition, prolonging the incubation of apoptotic cells for three days. Already 24 hours after apoptosis induction, soluble cytoplasmic proteins like lactate dehydrogenase (LDH) started to leak into the extracellular medium, and after 64 hours the entire complement of the protein was released. In contrast, HMGB1 was mostly retained associated with nuclear remnants, and in spite of the leaky membranes no significant release into the extracellular medium was observed (**Fig. 19**).

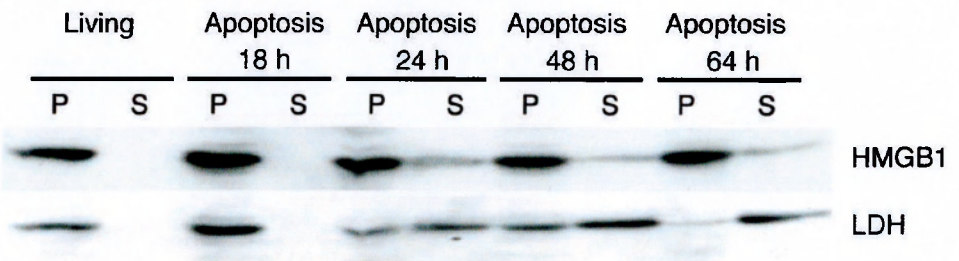

Fig. 19 Kinetics of protein release from cells undergoing apoptosis. Late during apoptosis, the integrity of the plasma membrane is lost, and soluble proteins (like lactate dehydrogenase, LDH) are leaked out to the medium (S). Even under conditions of extensive LDH leakage, most HMGB1 is retained associated to the cell remnants (P).

3.5 Necrotic cells release HMGB1

Since HMGB1 appeared to be tightly bound to the chromatin of cells undergoing *programmed* death, I analysed what happens in the case of *unprogrammed* cell death. Whereas apoptosis is an active process where a cell "decides" to suicide, necrosis occurs upon mechanical cell damage, trauma or poisoning. Necrosis differs from apoptosis biochemically, as it is a passive process where no protein is newly synthesised or modified, and morphologically: cells become rich with swollen and empty bodies containing disrupted organelles, nuclear chromatin is dispersed, and the integrity of the plasma membrane breaks down, making all the soluble proteins leak into the extracellular medium.

We forced HeLa cells into necrosis by ATP depletion, treating them with combinations of ionomycin and the mitochondria uncoupler CCCP, or deoxyglucose and azide. The number of cells undergoing necrosis was scored morphologically, and when it approached 50%, the supernatant was collected. A fraction of the treated cells was fixed and immunostained for HMGB1, the rest was analysed by Western blot, alongside with the extracellular medium. No HMGB1 was found associated with the remnants of necrotic cells, and a significant amount of the protein was recovered in the medium without any need for treatment with detergents (**Fig. 20**). Thus, as soon as the plasma membrane of the necrotic cells loses its integrity, HMGB1 diffuses out of the nucleus, and leaks into the extracellular medium.

Fig. 20 Chromatin association of HMGB1 in necrotic HeLa cells. HMGB1 was not associated to the chromatin of necrotic cells, even in absence of NP-40 permeabilisation. The amount of HMGB1 recovered in the medium was proportional to the number of necrotic cells (about 50%). Scale bars, 6 μm.

Taking together, the data obtained on apoptotic and necrotic cells lead to the conclusion that, upon cell death, HMGB1 behaves in opposite ways depending on how the cell dies. When sudden and unprogrammed death occurs, HMGB1 is passively released out of the cells, whereas in case of programmed cell death HMGB1 is firmly retained in the nucleus, even after autolysis of the cell.

3.6 Mobility of HMGB1 within the nuclei of living cells

The first set of experiments indicated that HMGB1 has three states of association with chromatin in living and dead cells: loose binding in living cells (both interphase and mitotic), no binding in necrotic cells, and tight binding in apoptotic cells.

In living interphase cells, at least a fraction of HMGB1 must bind to DNA, as we can detect the biological effects of this binding, for instance in terms of enhanced transcription. Nevertheless, this association is not stable and even upon detergent extraction we cannot distinguish between the soluble and the chromatin-associated pools. In mitosis, however, there is a clear spatial distinction between chromatin and cytoplasm, and in this condition it is evident that the majority of HMGB1 is bound to the chromosomes, and only a smaller fraction diffuses in the cytoplasm. This incomplete chromatin association in living cells could be due to the existence of two different HMGB1 pools, or a fast equilibrium of a single pool between two states. To discriminate between these possibilities, I probed the dynamic properties of HMGB1-GFP within mammalian nuclei with photobleaching techniques.

3.6.1 Photobleaching analysis

Photobleaching techniques are non-invasive microscopy methods which reveal the dynamics underlying the steady-state distribution of a fluorescently tagged protein in living cells. A fluorophore within a small region is irreversibly destroyed with a high intensity laser pulse. After bleaching, the labelled protein is photochemically altered, so that it no longer fluoresces, but otherwise retains completely its biological activity (Tsien, 1995). The exchange between the bleached and unbleached populations of fluorophore is then monitored, and used as an indicator of the overall mobility of the

protein. Two commonly used variations of this analysis, yielding complementary types of information, are fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP).

In FRAP, a small area in a cell expressing the fluorescent protein of interest is bleached once using a short laser pulse. Bleaching generates a region devoid of fluorescence signal, but if the molecule moves within the cell, the influx of unbleached molecules into the bleached area leads to a progressive recovery of fluorescence. The recovery kinetics depends on the exchange of molecules in the bleached region, and thus can be used to quantify the mobility of the protein **(Fig. 21A)**.

In FLIP, a region of interest is repeatedly bleached, and the loss of fluorescence from outside the bleached region is monitored, by imaging after each bleach dose. FLIP studies are particularly useful when the protein is not uniformly distributed but concentrated in defined intracellular sites, because it allows flux between populations of the fluorophore localised in different regions to be studied. In this case, the bleaching is made in the vicinity of the structure where the protein is concentrated. This creates a favourable situation for assessing the departure kinetics of molecules from that site into the surrounding zone in which they prevail at a lower steady state concentration, and this is observed as a loss of fluorescence from the region adjacent to the bleached spot **(Fig. 21B)**.

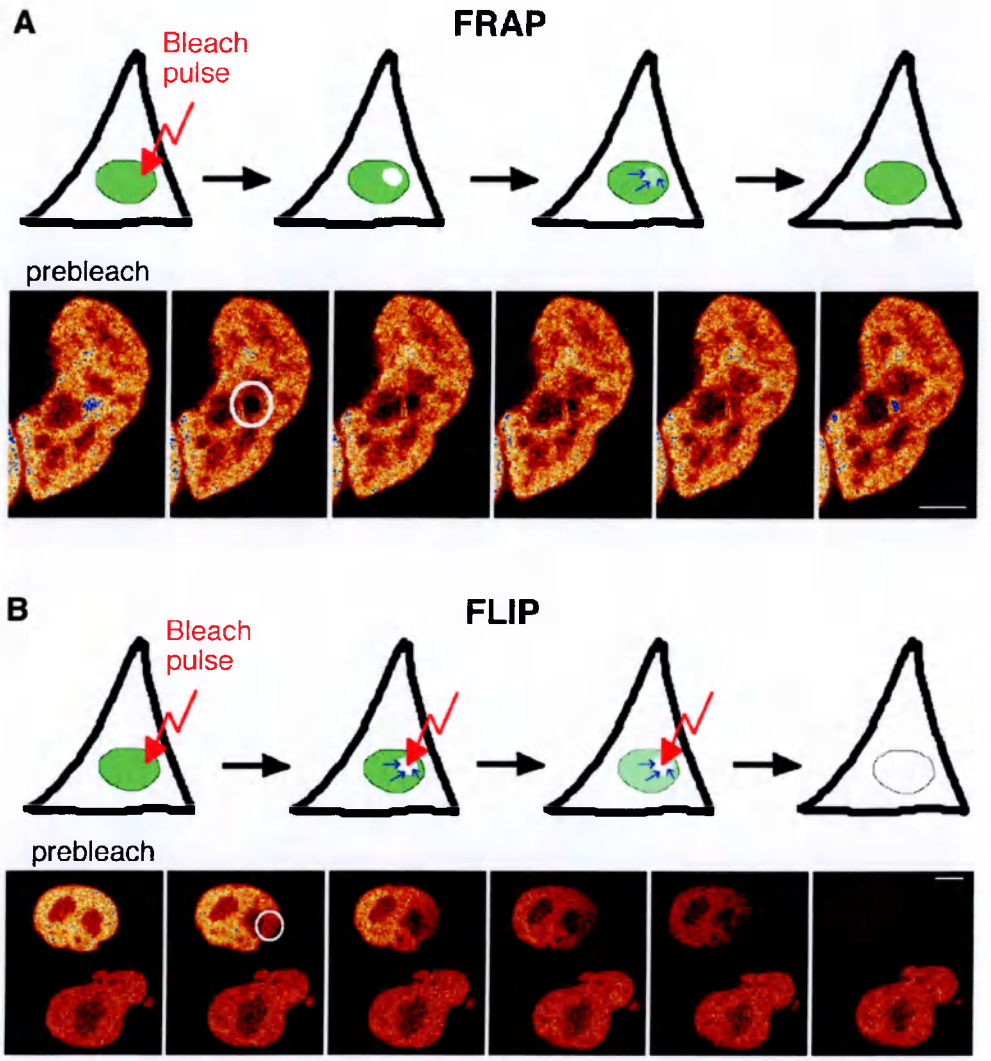

Fig. 21 ***In vivo*** **photobleaching techniques: FRAP and FLIP.** Scale bars, 2.5 μm.

A. In fluorescence recovery after photobleaching (FRAP), the fluorescence intensity in a small area after bleaching by a short laser pulse is measured using time-lapse microscopy. The recovery kinetics of mobile molecules are dependent on the mobility of the proteins. Immobile proteins show no recovery. Example of FRAP analysis performed on HeLa cells expressing a GFP-fusion to histone H1. In the bleached spot the fluorescent signal is lost after the laser pulse, but it is progressively recovered, thanks to the movement of the protein.

B. In fluorescence loss in photobleaching (FLIP), the fluorescence intensity is measured in a small area after repeated bleaching of a region that is distant from this area. The rate of loss of fluorescence signal is dependent on the mobility of the protein. Immobile proteins show no loss. Example of FLIP analysis performed on HeLa cells expressing GFP-fusion to the transcription factor NF1. After repeated bleaching of a small spot, the fluorescence signal is lost also outside that region, until all the fluorescence in the nucleus is exhausted.

The recovery of fluorescence in FRAP and the loss of fluorescence in FLIP are measured as a function of time using time-lapse microscopy, generating graphs like those shown in **Fig. 22**, which allow different biophysical parameters of the labelled molecule to be estimated. From FRAP curves we can easily determine the mobile and immobile fractions of the protein: the mobile fraction is the proportion of fluorescence that is regained, whereas the difference between the initial and final fluorescence corresponds to the immobile fraction. The recovery curve also gives a rough indication of the maximal time spent by the molecule in the same site before moving away (maximal residence time), which corresponds to the time when the recovery curve reaches the plateau. An additional parameter easily estimable by means of FRAP analysis is the diffusion coefficient of the protein, which allows to quantify the effective speed of the molecule. On the other hand, the rate of loss of fluorescence in FLIP experiments contains information on the dissociating flux of the protein from a particular site, and in combination with kinetic modelling allows the determination of relative rate constants for association and dissociation from compartments (Phair and Misteli, 2001).

FRAP and FLIP experiments measure apparent, not absolute, mobility, which is a combination of the diffusional mobility and the specific biological properties of a protein. In case of energy-independent transport, the most important factor which affects the speed of a molecule is the network of interactions established with cellular components which are relatively more immobile, such as cytoskeleton, membranes or chromatin. For chromatin binding proteins, the overall mobility is determined by their chromatin binding properties, therefore photobleaching methods can be used as a quantitative assay for their binding to chromatin *in vivo*.

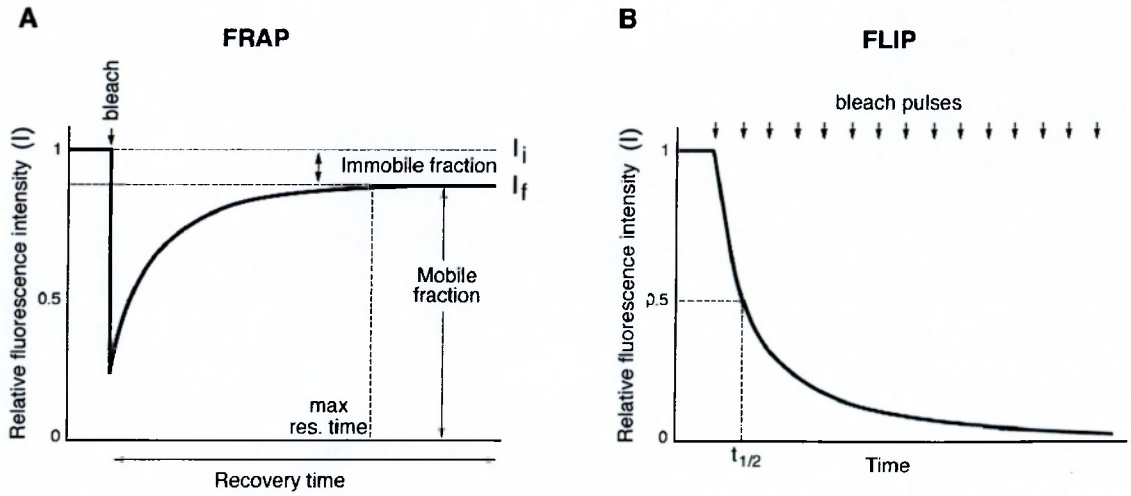

Fig. 22 Quantitative analysis from FRAP and FLIP.

A. An idealized plot of fluorescence intensity (I) as a function of time shows the parameters of a quantitative FRAP experiment. The bleach region is monitored during a pre-bleach period to determine the initial intensity I_i. This region is bleached using high-intensity illumination (intensity is not plotted during the bleach), and recovery is monitored immediately after the bleaching, until I reaches a final value I_f, when no further increase can be detected. Some methods calculate the effective diffusion coefficient, D_{eff}, directly from the time to reach half final intensity; the mobile fraction and the maximal residence time of the protein can also be measured. To determine all the parameters as accurately as possible, I must be corrected for the background intensity, and the amount of total fluorescence removed by the bleach.

B. An idealized plot of fluorescence intensity (I) as a function of time shows the parameters of a quantitative FLIP experiment. A region distant from the bleached area is monitored during a pre-bleach period to determine the initial intensity I_i and after each round of bleaching. The loss of fluorescence is monitored until I reaches a final value, when no further decrease can be detected. In combination with computational modelling the kinetics of loss of fluorescence allow the quantitation of biophysical properties of molecules and processes.

3.6.2 FRAP analysis on HMGB1 in living cells

I performed FRAP analysis on HeLa cells expressing HMGB1-GFP. A high-powered laser pulse of 250 ms was pointed in nucleoplasmic spots of transiently transfected cells and recovery of fluorescence was monitored starting 1.6 seconds after the laser pulse. Unexpectedly, after the bleaching, a minimal loss of HMGB1-GFP was observed in the selected areas, detectable only by pixel analysis, and the total fluorescence in the entire nucleus appeared slightly reduced (**Fig. 23A,C**). This result can only be explained by the rapid exchange of bleached protein in the target spot with unbleached protein from the surrounding areas, which recovers most of the fluorescence before the first image collection. Indeed, if the same experiment is done in paraformaldehyde-fixed cells in order to block the protein movement, a clear dark spot appears after the bleaching, and no recovery at all is observed, confirming that the minimal loss of fluorescence in living cells is due to the rapid movement of HMGB1-GFP (**Fig. 23B,C**).

The quantitative analysis shows that in living cells 80% of the fluorescent signal is already regained after 1.6 seconds, and the recovery is essentially complete after 4 seconds (**Fig. 23C**). This value corresponds to the maximal residence time of HMGB1 on chromatin, and gives a clear idea of the dynamic behaviour of the protein in the nucleus. This rapid movement is energy-independent, as inhibition of ATP synthesis did not affect the kinetics of the process.

The mobility of HMGB1-GFP appeared to be much faster than that of other nuclear proteins used as controls (**Fig. 23 D**). HMGN1-2 are closely related chromatin proteins belonging to the HMGN subclass of the high mobility protein family, different from the HMGB one (Bustin et al., 1990; Bustin, 2001). They bind to nucleosomes and are thought to alter the higher order chromatin structure, modulating the access of

transcriptional regulators to DNA. Confirming published data (Phair and Misteli, 2000), I found that also HMGN1-2 are highly mobile in the nucleus, but the maximal residence time of these proteins is about 8 fold longer compared to HMGB1.

I also analysed the mobility of the linker histone H1. H1 is also believed to be involved in chromatin organisation by stabilising higher order structures, and it has been considered for a long time a sort of HMGB1 *alter ego* (Dimitrov et al., 1994, 1993; Ner and Travers, 1994), with overlapping functions. As mentioned in the introduction (paragraph 2.5), previous findings had already argued against this view, ruling out the possibility of a shared structural role for HMGB1 and histone H1 (Falciola et al., 1994; Spada et al., 1998). Photobleaching experiments confirmed those data. Again in accordance with published results (Misteli et al., 2000; Lever et al., 2000), I found that also H1-GFP is not static and is continuously exchanged between chromatin regions; however the mobility of the protein is extremely low, indicating a strong association to DNA. The maximal residence time of H1, about 4 minutes, is much longer than the 4 seconds of HMGB1, and suggests completely different chromatin binding properties for the two proteins.

Only unfused GFP, which is usually used as a standard in this type of assays, appeared to be faster than HMGB1-GFP, recovering its fluorescence signal to 98% of the pre-bleach value within 1 s. This result was expected as unfused GFP does not functionally interact with any molecule within the nucleus and its diffusion mobility is not slowed at all.

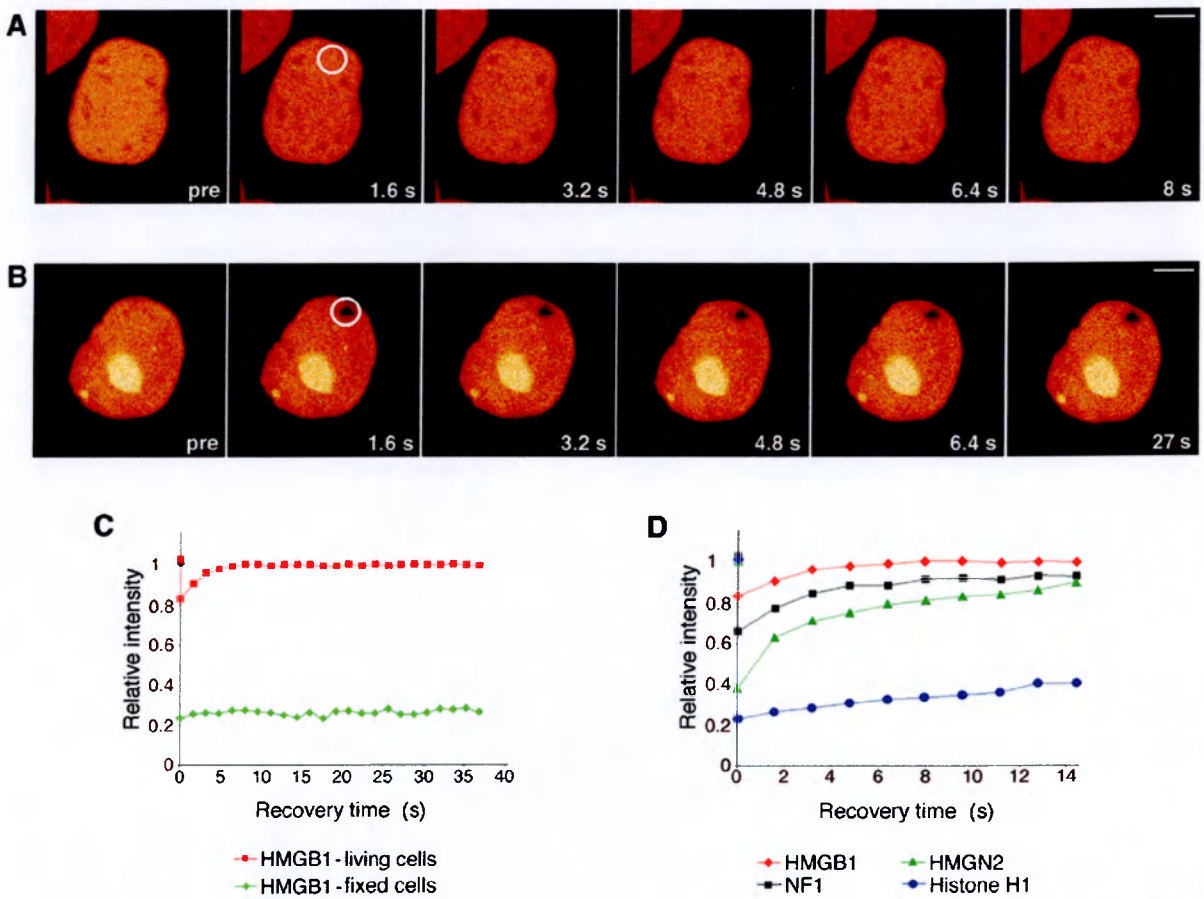

Fig. 23 HMGB1 dynamics in interphase cells: FRAP ananlysis. Scale bars, 2.3 μm.

A. FRAP in living cells. A HeLa cell expressing HMGB1-GFP was imaged before and during recovery after a single bleaching of a nuclear area (circle) for 250 ms. Images, shown in pseudocolours, were taken at the indicated times after end of the bleach pulse. Most of the fluorescence in the bleached spot was recovered already after 1.6 s, indicating a very rapid movement of the protein.

B. FRAP in fixed cells. Cells were fixed with PFA and analysed as described in A. When the movement of HMGB1-GFP was blocked, no recovery was observed in the bleached spot.

C. Kinetics of fluorescence recovery in FRAP on HMGB1-GFP. In living cells, the fluorescence signal was 80% recovered after 1.6 s already, and completely recovered after 4 s. In fixed cells, the fluorescence signal decreased to the background level after the bleaching, and, afterwards, the recovery curve was completely flat.

D. Kinetics of fluorescence recovery in FRAP on GFP-fusions to HMGN2, NF1 and histone H1. The kinetics of fluorescence recovery for all the other nuclear proteins used as controls was slower compared to HMGB1-GFP.

3.6.3 FLIP analysis on HMGB1 in living cells

I verified the high mobility of HMGB1 also by FLIP analysis. In interphase cells, after 2 minutes of repeated bleaching in the same nucleoplasmic spot, the fluorescence signal was lost in the entire nucleus confirming that HMGB1-GFP moves very rapidly (**Fig. 24A**). In contrast, fluorescence loss in HeLa cells expressing GFP fusions to chromatin proteins HMGN1 and HMGN2, transcription factor NF1, or histone H1 was significantly slower (**Fig. 24C**).

I extended the analysis to mitotic cells, where HMGB1 is mostly associated with chromosomes, but also present in the cytoplasm, at a lower concentration. This is the ideal situation to study the binding of HMGB1 to chromatin, since DNA is clearly separated from the cytoplasm, and it is possible to discriminate between soluble or DNA-bound molecules. In FLIP experiments, repeated bleaching of cytoplasmic HMGB1-GFP led to rapid and complete loss of fluorescence both from condensed chromosomes and from the cytoplasm, with comparable kinetics (**Fig. 24B,D**). This result indicates that also in mitosis all HMGB1 molecules are highly dynamic and rapidly shuttle between the cytoplasm and chromosomes. The concentration of HMGB1 on chromosomes therefore is not due to a static binding, but rather is the result of a steady-state in which HMGB1 rapidly and continuously associates and dissociates on DNA. In these experiments, the laser was pointed on a cytoplasmic region, thus the loss of fluorescence on chromosomes is a measure of the departure flux of HMGB1 from DNA. This flux depends on both association/dissociation kinetics on DNA and the diffusion rate of the molecules. Instead, the loss in the cytoplasm is dependent only on the diffusion rate of the protein. The curves obtained in both cases are identical, and this implies that the binding of HMGB1 to chromosomes does not slow significantly its diffusion movement, further confirming the transient nature of this association.

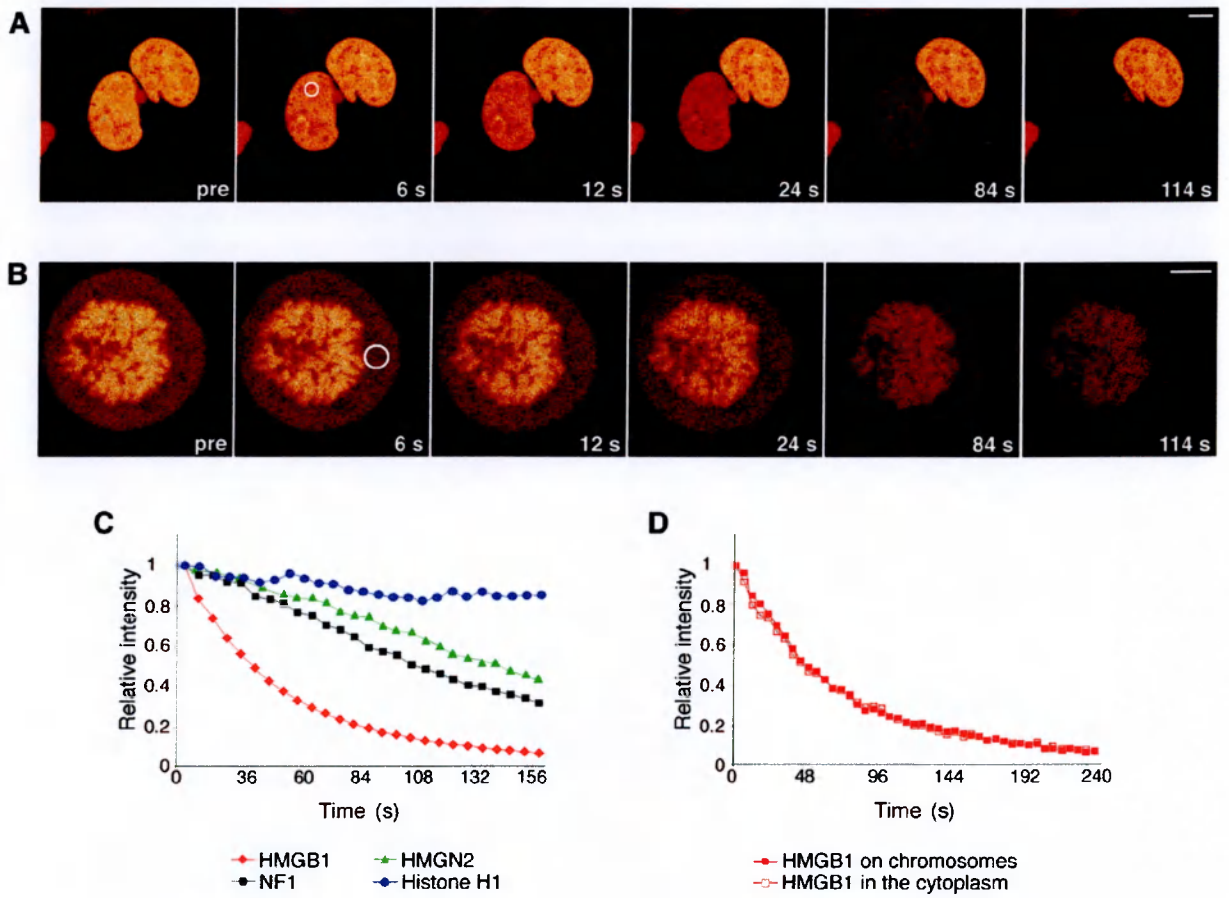

Fig. 24 HMGB1 dynamics in interphase and mitotic cells: FLIP analysis. Scale bars, 2.3 μm.

A. FLIP on interphase cells. The area indicated by a circle was repeatedly bleached for 200 ms, and cells were imaged between bleach pulses. After 2 minutes almost all the fluorescence signal was lost from the entire nucleus, showing a high intranuclear mobility of HMGB1-GFP. A neighbouring cell nucleus was not affected by the repeated bleach pulses.

B. FLIP on a cytoplasmic region of a mitotic cell. The loss of fluorescence was fast and complete in the whole cell, including condensed chromosomes. There is interchange between chromosomal and cytoplasmic pools of HMGB1-GFP, and a rapid dissociation of the protein from condensed chromatin.

C-D. Kinetics of fluorescence loss in FLIP. In interphase cells HMGB1-GFP fluorescence was lost much faster than that of other nuclear proteins (**C**). In mitotic cells, bleaching was executed on a spot in the cytoplasm, and quantification was done both on a different spot in the cytoplasm, or on a spot on the condensed chromosomes. The kinetics are identical (**D**), indicating a rapid turnover of HMGB1-GFP on condensed chromatin.

Photobleaching analysis confirmed the indication derived from detergent extraction experiments, proving that in living cells HMGB1 is highly dynamic in its binding to chromatin, both condensed and decondensed, and rapidly roams throughout the nucleus, establishing transient interactions with DNA.

3.7 Mobility of HMGB1 within the nucleus of apoptotic cells

The permeabilisation experiments suggested that in apoptosis HMGB1 might be more stably associated with chromatin. I tried to confirm this by measuring the mobility of HMGB1 in apoptotic cells. FRAP and FLIP analysis performed on TNF-α treated cells revealed that HMGB1-GFP is completely immobile in apoptotic cells, not unlike in chemically fixed cells (FRAP, **Fig. 25A,C**; FLIP, **Fig. 25B,D**). In FRAP experiments, after the laser pulse the fluorescence signal was totally lost in the bleached region, and no successive recovery was observed. In FLIP experiments, repeated bleaching in the same area did not lead to loss of fluorescence in the surrounding regions. These results indicate that in apoptosis HMGB1 freezes onto chromatin and the binding is so strong that virtually no molecule is able to diffuse within the nucleus.

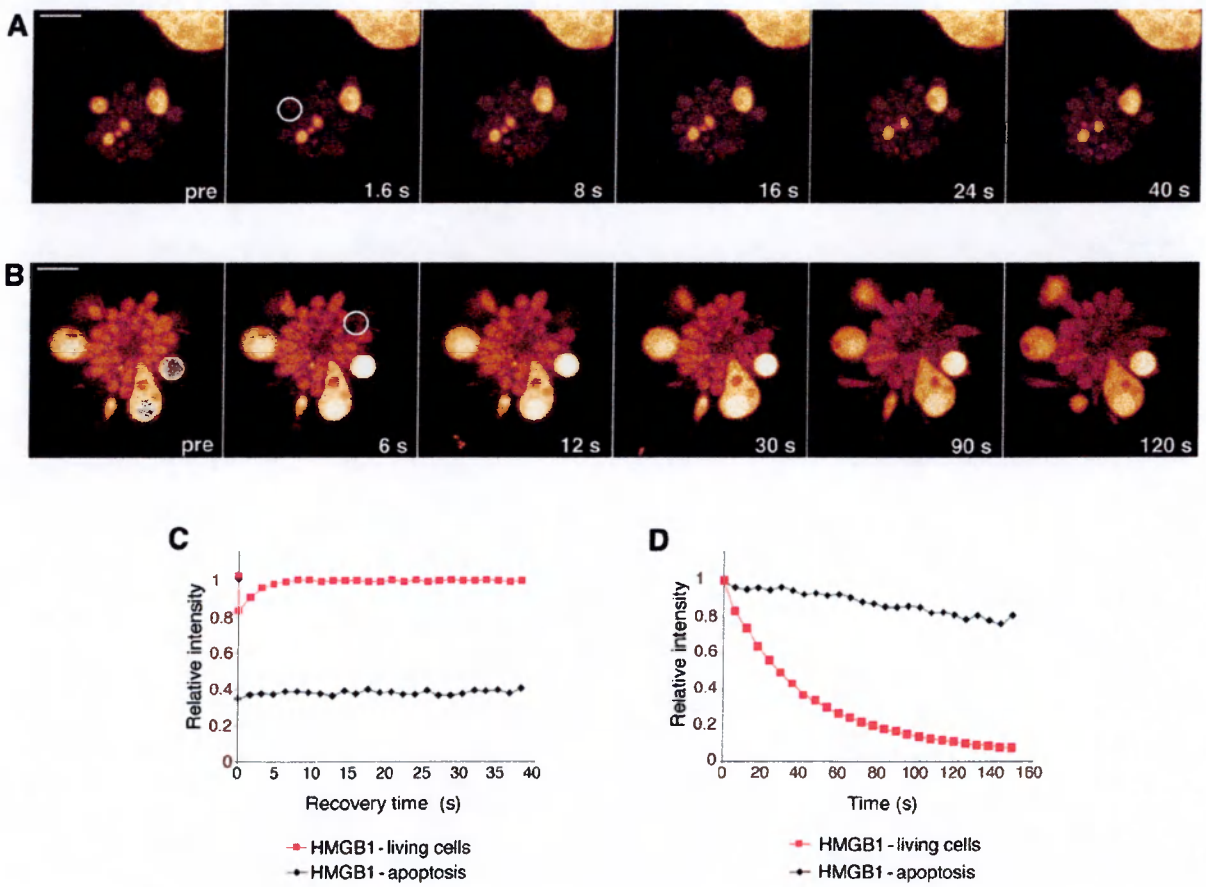

Fig. 25 Photobleaching analysis in apoptotic cells. Scale bars, 3.7 μm.

A. FRAP. A HeLa cell expressing HMGB1-GFP, forced to undergo apoptosis, was imaged as indicated in the legend to Fig. 22. After the laser pulse, no recovery of the fluorescence signal was observed in the bleached region (circle), indicating a complete blockade of HMGB1 movement.

B. FLIP. The spot indicated by a circle was repeatedly bleached for 200 ms in an apoptotic cell. Two minutes after the first pulse, no loss of fluorescence was observed outside the bleached region, confirming that HMGB1 was not moving within the cell.

C-D. Kinetics of fluorescence recovery (FRAP) (**C**) and loss of fluorescence (FLIP) (**D**) of HMGB1-GFP in apoptotic HeLa cells as compared to living cells.

The blockade of HMGB1 mobility in apoptosis is specific, because the mobility of GFP-HMGN1, GFP-HMGN2, GFP-NF1, GFP-H1 and GFP alone is not reduced, or even increases, in apoptotic cells as compared with living ones (**Fig. 26**). Thus, the condensation and fragmentation of chromatin occurring during apoptosis does not impair protein mobility in general.

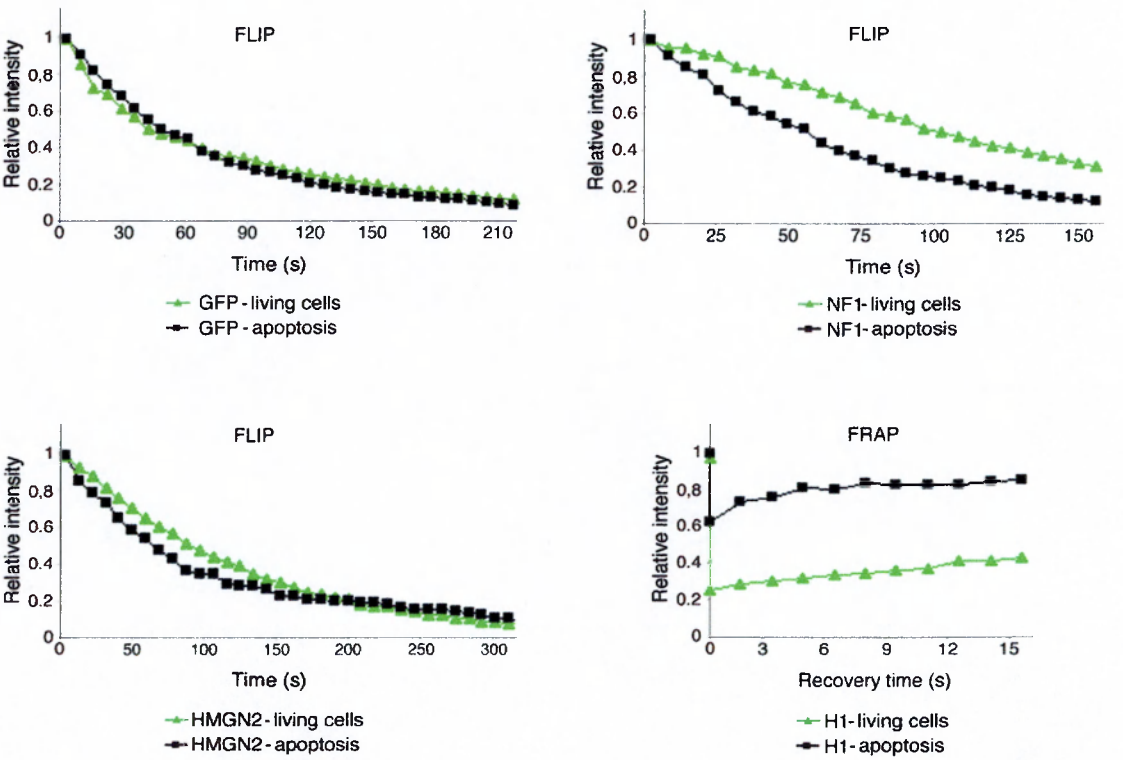

Fig. 26 Mobility of unfused GFP and GFP-fusions to HMGN2, NF1 and histone H1 in apoptotic cells. FLIP kinetics of unfused GFP, GFP-NF1 and GFP-HMGN2, and FRAP kinetics of GFP-H1 in apoptotic HeLa cells as compared to living cells. In apoptosis, the mobility of unfused GFP and HMGN2 did not change significantly, whereas NF1 and H1 showed even an increase in their mobility. The mobility of H1 was measured by FRAP because this method is more suitable than FLIP for the analysis of slow proteins.

3.8 Apoptosis in *Hmgb1*$^{-/-}$ cells

The specific tight association of HMGB1 with apoptotic chromatin suggested that HMGB1 might have an active role in the apoptotic process, presumably in modulating DNA structure. To check this hypothesis, I forced embryonic fibroblasts obtained from *Hmgb1*$^{-/-}$ and *Hmgb1*$^{+/+}$ mice into apoptosis, and compared their behaviour. Both cell lines were equally susceptible to apoptosis, and a morphological analysis did not show any evident defect in the chromatin content of apoptotic cells lacking HMGB1. These data suggest that the binding of HMGB1 to chromatin is not required for apoptosis, but rather is a consequence of the process.

Dramatic changes occur in the cell during apoptosis: post-translational modifications affect the activity of many proteins, large condensed chromatin bodies form in the nucleus and DNA is cleaved at internucleosomal sites. I tried to understand which of these processes is linked to the stable binding of HMGB1 to the chromatin of apoptotic cells.

3.9 Post-translational modifications of HMGB1 in living and apoptotic cells.

HMGB1 showed a differential affinity for chromatin in living and apoptotic cells. As a first step in trying to elucidate the molecular basis of this difference, I checked whether HMGB1 itself changes in apoptosis.

When cells undergo apoptosis, many proteins are post-translationally modified. A subset of them is irreversibly modified by proteolytic cleavage, the rest is covalently modified by phosphorylation, acetylation, methylation, or similar processes. In the first case, key players are apoptotic-specific proteases named caspases, a group of at least

nine cysteine proteases related to the cell death protein CED-3 in *C. elegans* (Kumar, 1995). Caspases are synthesised as inactive zymogens, which are activated during apoptosis upon proteolytic cleavage. Active caspases in turn cleave a wide range of cellular substrates containing a DXXD sequence, thereby either increasing or abolishing their activity (Thornberry et al., 1997).

HMGB1 contains a putative double site for caspase cleavage (DEEDEED) at the beginning of the acidic tail (**Fig. 27A**). The removal of this negatively charged region would presumably increase the affinity of the protein for DNA, thus I checked whether HMGB1 is cleaved by active caspases by comparing the molecular weight of the protein in living and apoptotic cells. Total extract from control and either TNF-α-treated 3T3 or HeLa cells were analysed by Western blot, but no change was seen in apoptosis either in the amount of HMGB1 or in its molecular weight, ruling out the possibility that HMGB1 was cleaved by active caspases (**Fig. 27B**).

I then checked whether covalent modifications could affect the chromatin binding properties of HMGB1 in apoptosis. Old data reported that HMGB1 is acetylated, and that this modification can modulate its DNA binding activity (Johns, 1982; Dimov et al., 1990). Tiziana Bonaldi in our lab has recently characterised the acetylation pattern of HMGB1, showing that several isoforms with different levels of acetylation are present in living cells. Acetylation consists of the addition of acetyl groups (CH_3-CO-) to the ε amino group of lysine residues, and leads to the neutralisation of a positive charge and modulation of the isoelectric point (pI) of the protein. The process is reversible and specific enzymes, acetylases and deacetylases, are responsible, respectively, for addition or removal of acetyl groups. Modulation of the total charge of a protein by acetylation can dramatically affect its ability to interact with other molecules, and contributes to the regulation of its activity (Prives C, 2001; Hung HL,

1999). It is possible that a deacetylation of HMGB1 may increase its affinity for DNA, as the net charge of the protein becomes more positive. Therefore, deacetylating HMGB1 during apoptosis could be a way to stabilize its binding to chromatin. To test this possibility, I compared the acetylation status of HMGB1 in total extracts obtained from living and apoptotic cells, by means of two-dimensional gel electrophoresis (2D PAGE). Both in living and apoptotic cells HMGB1 was resolved into several isoforms, appearing as two spots at the same molecular weight coordinate but corresponding to different isoelectric points. The difference between the two isoforms is the number of modified lysines and the more basic isoform represents the less acetylated one. However, no shift towards basic pI was observed for HMGB1 in apoptotic cells as compared to living ones, meaning that even the acetylation status of the protein is not modified during programmed cell death (**Fig. 27C**).

In conclusion, this biochemical analysis shows that no changes in HMGB1 were detected in apoptosis, and its tight association to chromatin is unlikely to be due to post-translational modifications which increase its affinity to DNA.

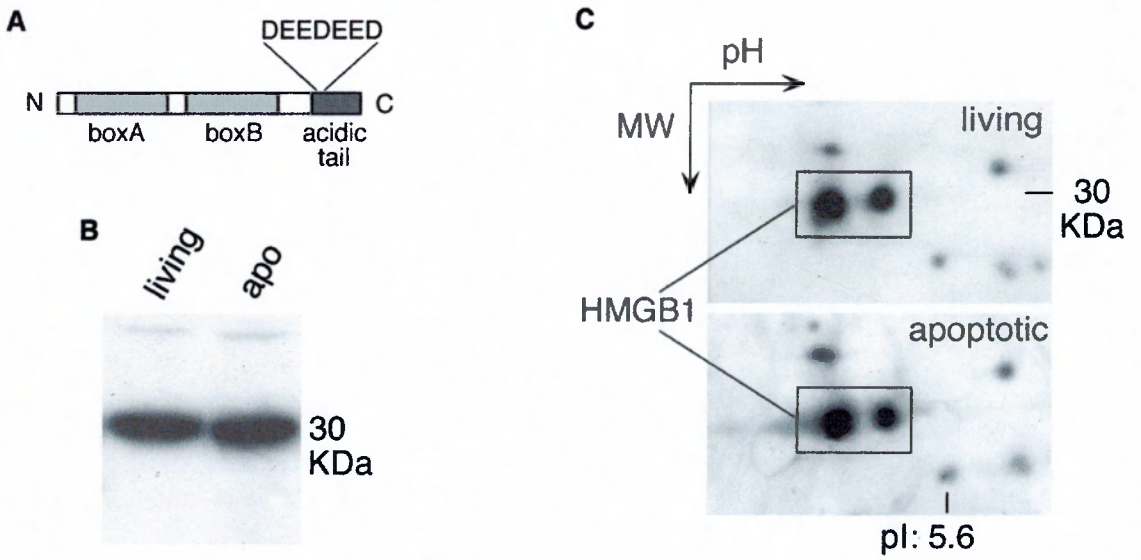

Fig. 27 HMGB1 is not differentially modified in living and apoptotic cells.

A. Schematic representation of the structure of HMGB1 and localisation of the putative cleavage site for active caspases.

B. Monodimensional gel electophoresis and Western blot with anti-HMGB1 antibodies on total extracts obtained from living and apoptotic HeLa cells. The molecular weight of the protein does not change in apoptosis, indicating that HMGB1 is not cleaved by active caspases.

C. Two-dimensional gel electrophoresis and Western blot with anti-HMGB1 antibodies on total extracts obtained from living and apoptotic HeLa cells. Multiple acetylated forms of HMGB1 are visible, but no difference in the pI is detectable between the two samples, meaning that the protein is not differentially acetylated during apoptosis.

3.10 Chromatin changes in apoptosis: effects on HMGB1 and its binding to DNA

During apoptosis, both nuclear architecture and chromatin content undergo dramatic alterations. Because no variation in HMGB1 was detected in apoptosis, I focused on these cellular changes to explain the different chromatin binding properties of HMGB1 in apoptotic cells. In particular, I checked whether apoptotic chromatin represents a better substrate for HMGB1, compared with the chromatin of living cells. Chromatin condensation and DNA cleavage at internucleosomal sites are the most recognizable markers of apoptosis, even though not many data are available on the molecular events underlying these processes. Electron microscopy on isolated apoptotic chromatin has shown that the formation of large condensed chromatin domains is accompanied by a specific conformational transition at the nucleosome level (Allera et al., 1997). Already in the early stages of the process, the core particles appear to be tightly packed face-to-face in smooth 11-nm filaments, at variance with their radial disposition around the solenoid axis in the 30-nm fiber (Bednar et al., 1998). These filaments progressively fold to generate a closely woven network, which finally collapses, producing dense apoptotic bodies (Allera et al., 1997). Simultaneously to chromatin condensation, genomic DNA is cleaved at internucleosomal sites. Two stages of DNA degradation are observed. Early in the process, the DNA is cleaved to high molecular weight fragments, probably reflecting a large-scale domain organisation of the chromatin. Later on, the chromatin is cut into shorter fragments, generating oligo- and mononucleosomes (Allera et al., 1997).

I tried to understand whether these alterations in the chromatin content of apoptotic cells could be responsible for the stable binding of HMGB1. *Hmgb1*$^{-/-}$ cells offered an opportunity to test this hypothesis. *Hmgb1*$^{-/-}$ fibroblasts were treated with TNF-α and cycloheximide, and apoptotic cells detaching from the plate were recovered.

This cell population, and a control population of non-apoptotic, interphase *Hmgb1*$^{-/-}$ fibroblasts, were then permeabilised with 0.5% NP-40, and exposed to HMGB1 produced in *E. coli* and labelled with Cy5 fluorophore. Microscopic examination showed that HMGB1 bound to apoptotic nuclei, but not to non-apoptotic ones (**Fig. 28A**).

The differential binding of HMGB1 to apoptotic vs. non-apoptotic nuclei was also confirmed biochemically (**Fig. 28B**). Permeabilised apoptotic and non-apoptotic *Hmgb1*$^{-/-}$ fibroblasts were incubated with unmodified recombinant HMGB1 and centrifuged through a discontinuous sucrose gradient. The nuclear pellet, containing chromatin free from membrane debris, together with associated HMGB1, was then recovered and subjected to electrophoresis. Again, HMGB1 appeared associated with the apoptotic nuclei, but not with non-apoptotic ones.

These experiments indicated that, upon apoptosis, chromatin undergoes some chemical or structural transition that makes it susceptible to HMGB1 binding. The nature of HMGB1 itself, whether endogenous or made in bacteria, chemically modified by the addition of fluorophores, or fused to GFP, appears to be irrelevant.

I next investigated the nature of the chromatin modification allowing the stable binding of HMGB1.

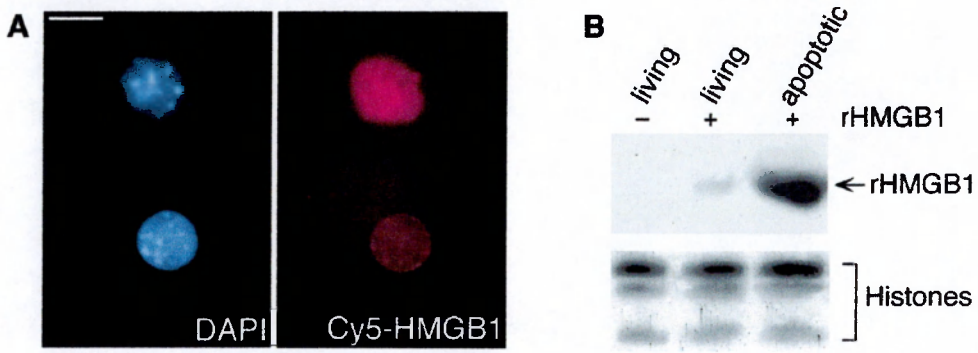

Fig. 28 Chromatin changes occurring in apoptosis create stable binding sites for HMGB1. Scale bar, 9.5 μm.

A. *Hmgb1*$^{-/-}$ fibroblasts were permeabilised and incubated with 1 μM recombinant Cy5-labelled HMGB1. HMGB1 stuck to the apoptotic nucleus (upper) but not to the non-apoptotic one (lower).

B. *Hmgb1*$^{-/-}$ fibroblasts, either undergoing apoptosis or in control untreated cultures were permeabilised, mixed with recombinant unlabelled HMGB1 and centrifuged through a sucrose gradient. Nuclei recovered from the bottom of the tubes were then subjected to SDS-PAGE; histones were visualized by Coomassie staining, and HMGB1 by immunostaining.

3.11 Fragmentation of DNA

Because HMGB1 binds tightly to reconstituted mononucleosomes *in vitro*, I tested whether the fragmentation of chromatin to oligo- and mononucleosomes occurring *in vivo* in the latest stages of apoptosis would provide stable binding sites for HMGB1. With this aim, I tried to dissociate apoptosis from DNA degradation.

To date, the best characterized endonuclease involved in this process in vertebrates is the Caspase-activated DNase (CAD, also named as CPANN or DFF40) (Enari et al., 1998; Halenbeck et al., 1998; Liu et al., 1998). CAD is a 40 KDa endonuclease which in living cells is found associated with a second polypeptide of 45 KDa named Inhibitor of CAD (ICAD, or DFF 45) (Liu et al., 1997). ICAD is both a folding chaperone and an enzymatic inhibitor of CAD. If CAD is translated in the absence of ICAD it adopts an inactive conformation and is catalytically inert (Enari et al., 1998). If CAD is translated in the presence of ICAD, it adopts an active conformation but is catalytically inactive due to the binding of the ICAD inhibitor. Upon induction of apoptosis, ICAD is cleaved in two fragments by caspase-3, and releases active CAD (Liu et al., 1997; Sakahira et al., 1998). DNA cleavage during apoptosis can be totally blocked by inhibiting CAD activity through the expression of a caspase-resistant ICAD. It has been shown however that also overexpression of wild-type ICAD can completely block DNA degradation upon apoptotic stimuli, suggesting that it is possible to inhibit CAD, just maintaining a small, constant, excess of uncleaved ICAD.

HeLa cells were stably transfected with a construct expressing a tagged form of wild-type ICAD and treated with TNF-α and cycloheximide. The cells underwent apoptosis, showing the characteristic membrane blebbing and chromatin condensation, and most ICAD was cleaved by active caspases, so that no full-length protein was

detectable by Western blot (**Fig. 29A**). However, the small amount left was sufficient to completely inhibit the internucleosomal cleavage of chromosomal DNA, as verified by agarose gel electrophoresis (**Fig. 29A**). In contrast to control apoptotic cells which showed the characteristic DNA ladder, ICAD-overexpressing cells contained intact genomic DNA, exactly as living cells. I also confirmed the inhibition of DNA degradation at the single cell level. TUNEL staining allows visualization of fluorescent nuclei containing fragmented DNA, and is usually used to detect late apoptotic cells. Control apoptotic cells were all positive for TUNEL staining, whereas only a background signal was observed in apoptotic ICAD-overexpressing cells (**Fig. 29B**).

ICAD-overexpressing cells offered an opportunity to evaluate the effect of DNA degradation on HMGB1 binding to apoptotic chromatin, as in this system apoptosis occurs without DNA fragmentation. I therefore checked whether the association of HMGB1 with chromatin could be affected by preventing DNA fragmentation, but I found that HMGB1 bound equally stably to nonfragmented chromatin and to fragmented chromatin (**Fig. 29B**).

On the basis of this result, we concluded that fragmentation per se is not sufficient to create appropriate HMGB1 binding sites in apoptotic cells.

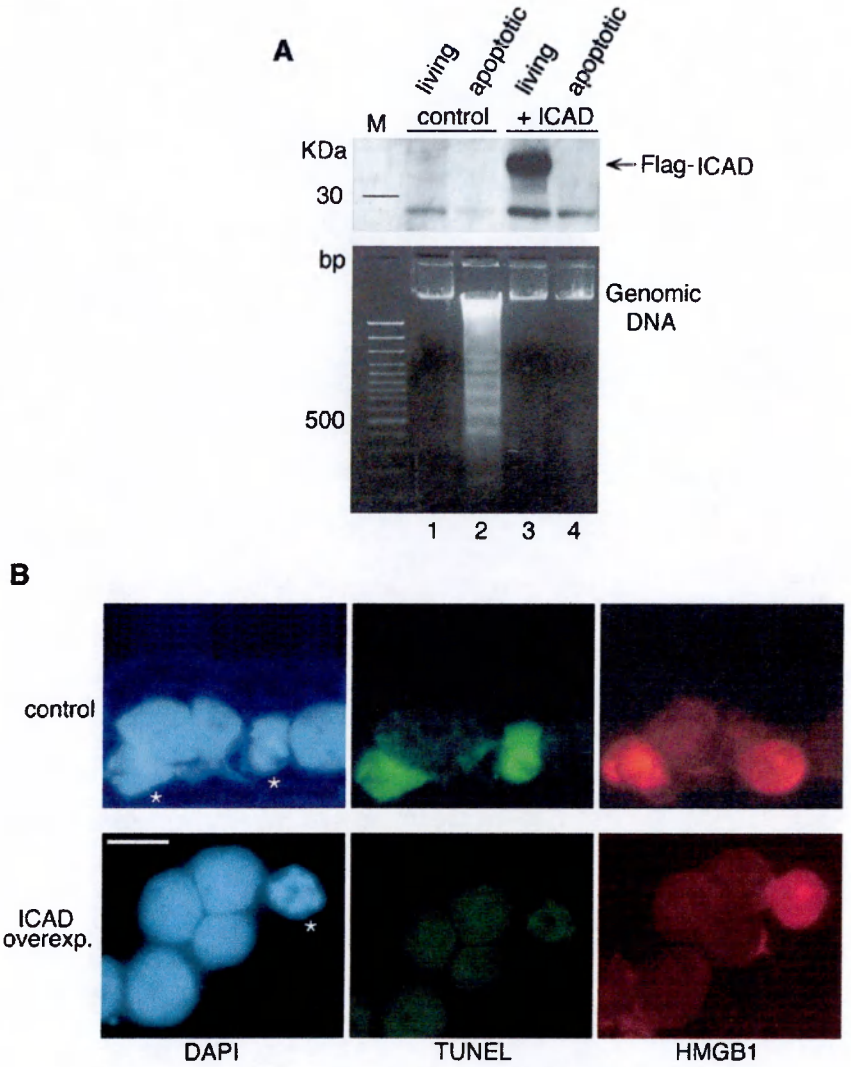

Fig. 29 DNA fragmentation is not responsible for HMGB1 binding to apoptotic nuclei. HeLa cells were stably transfected to overexpress a tagged form of ICAD.

A. ICAD-expressing and control cells were induced into apoptosis (apoptotic, lanes 2 and 4), or were mock treated (living, lanes 1 and 3). ICAD was visualized by immunoblotting with anti-FLAG antibodies. ICAD in apoptosis was cleaved by caspase, and lost the FLAG tag (lane 4, Western blot). The internucleosomal cleavage of chromosomal DNA was evident in apoptotic wild type cells (lane 2), but was inhibited in apoptotic ICAD-expressing cells (lane 4), as visualized by agarose gel electrophoresis.

B. Control and ICAD-expressing cells were treated with TNF-α, permeabilised, fixed and stained for DNA and HMGB1. While control apoptotic cells were positive for TUNEL staining, indicating genomic DNA fragmentation, apoptotic ICAD-expressing cells were TUNEL-negative confirming the inhibition of DNA degradation. Nevertheless, even in the absence of DNA cleavage, HMGB1 was firmly retained into the nucleus of the cell already showing chromatin condensation (white asterisk, lower pannel), as well as into the control apoptotic cells (white asterisks, upper panel). Scale bar, 10 μm.

3.12 Effect of TSA treatment

I next focused on the acetylation status of the apoptotic chromatin. Modulation of histone tail acetylation is one of the major mechanisms responsible for the remodelling of chromatin structure. The process is under the control of nuclear Histone Acetyl-Transferases (HATs) and Histone Deacetylases (HDACs), two families of enzymes which respectively add or remove acetyl groups at histones tail lysine residues. Histone acetylation destabilizes the chromatin fiber and creates an "open" chromatin configuration, making DNA accessible to transcription factors or other DNA binding proteins (Davie and Chadee, 1998). In contrast, deacetylation of histones tails facilitates chromatin condensation, and generates compact regions, which are usually trascriptionally incompetent.

The chromatin acetylation pattern can be altered by inhibiting either HATs or HDACs. General inhibitors for both families exist, as well as specific inhibitors for individual enzymes (Jung, 2001; Lau et al., 2000). Trichostatin A (TSA) is a general deacetylase inhibitor, and treatment of cells with this drug enhances the level of histone acetylation (Yoshida et al., 1990).

I added TSA to the medium of HeLa cells just prior to the induction of apoptosis and tested the effect of hyperacetylation of apoptotic chromatin on HMGB1 mobility. Both FRAP and FLIP analysis showed that HMGB1 binding to apoptotic chromatin was suppressed when cells were treated with TSA (**Fig. 30A,B**). The treatment totally reverted the blockade of HMGB1 movement in apoptosis and the protein diffused in the nucleus with kinetics almost identical to those measured in living cells (**Fig. 30C,D**). This result suggests that hypoacetylation of one or more chromatin components during apoptosis is required to allow HMGB1 binding.

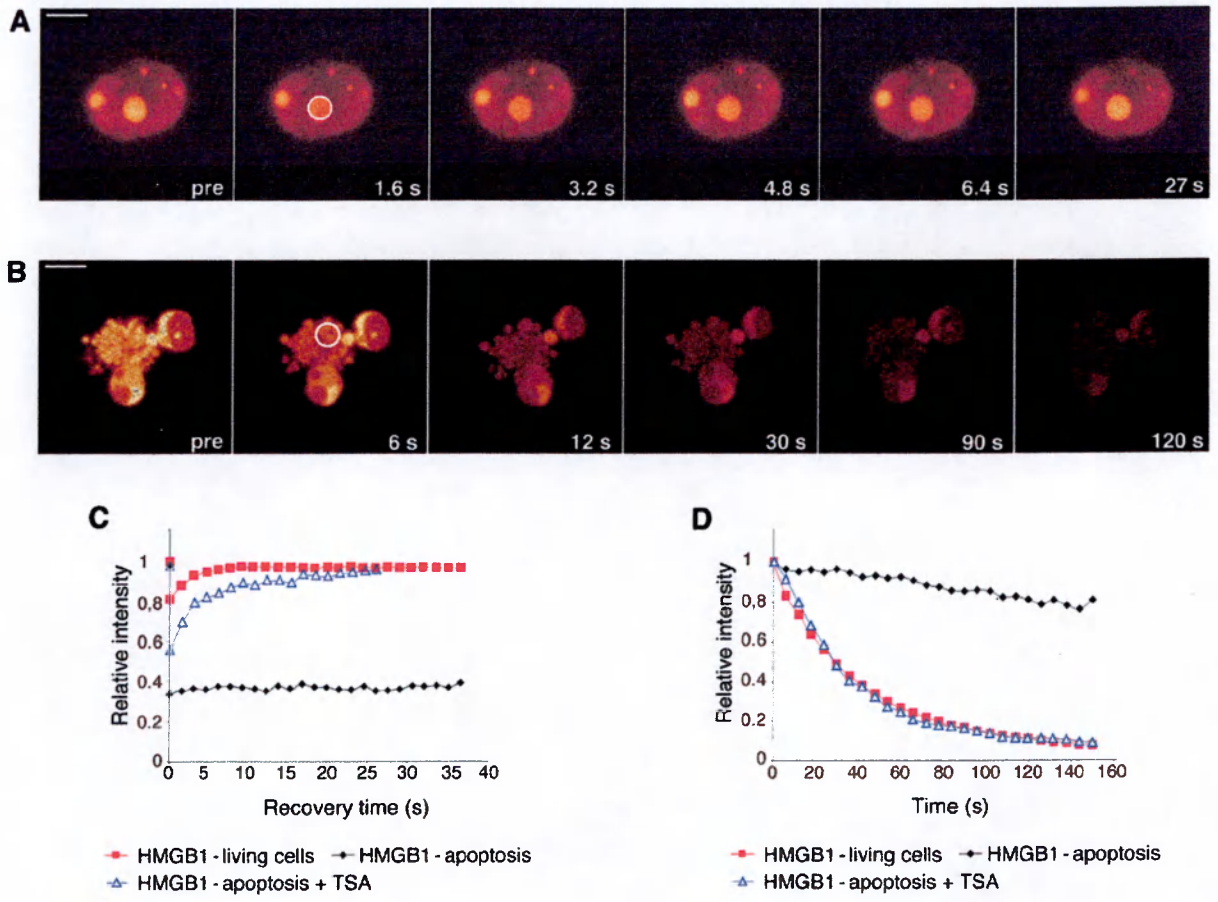

Fig. 30 Effect of TSA treatment on HMGB1 dynamics in apoptotic cells. Scale bars, 4.7 μm.

A. FRAP analysis on HeLa cells undergoing apoptosis in the presence of 200 ng/ml TSA. A cell was imaged before and during recovery after a single bleaching of a nuclear area (circle), as described in the legend to Fig. 22. Most of the fluorescence in the bleached spot was recovered already after 1.6 s, indicating that the HMGB1 movement in apoptosis was restored in the presence of TSA.

B. FLIP. A cell undergoing apoptosis in the presence of TSA was repeatedly bleached in the same spot (indicated by a circle), and imaged between bleach pulses. Complete loss of fluorescence was observed also outside the bleached area, confirming the restored mobility of HMGB1.

C-D. Kinetics of fluorescence recovery (FRAP) (**C**) and loss of fluorescence (FLIP) (**D**) of HMGB1-GFP in living HeLa cells, as compared to apoptotic cells in the presence and absence of TSA. TSA totally reverted the mobility blockade of HMGB1.

I then checked whether the acetylation status of histones changes in apoptosis. Nuclear extracts obtained from living and apoptotic cells were analysed by Western blot using antibodies that recognise acetylated lysine residues. Histone H4 from apoptotic chromatin appeared to be notably hypoacetylated in comparison to non-apoptotic chromatin, whereas no difference was observed in the acetylation status of histones H2A, H2B, H3 (**Fig. 31A**). On the contrary, when cells were treated with TSA, all core histones underwent a marked hyperacetylation even in apoptosis (**Fig. 31A**). H4 hypoacetylation was also confirmed using a polyclonal antibody raised specifically against the acetylated form of the protein (**Fig. 31B**).

In conclusion, histone H4 is deacetylated during apoptosis, but this deacetylation can be prevented by treating the cells with TSA, and upon treatment the binding of HMGB1 to chromatin is completely abolished.

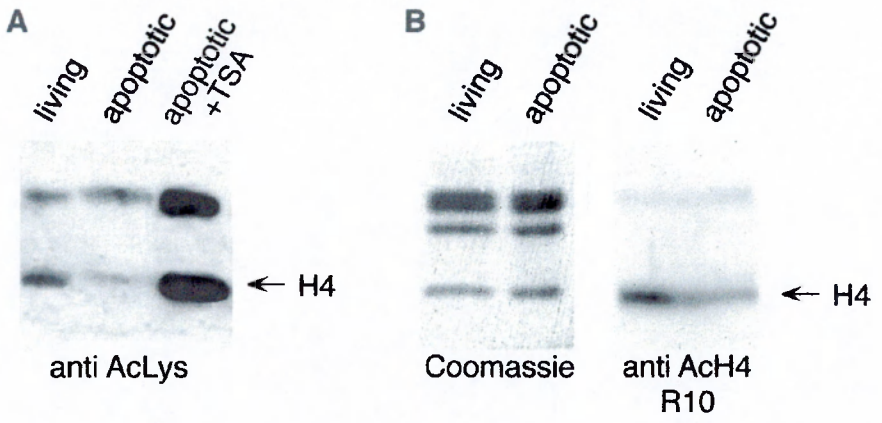

Fig. 31 Acetylation status of histones in apoptosis.

A. Total extracts from living cells and cells induced to apoptosis in the presence or in the absence of TSA were analysed by Western blot, using polyclonal antibodies specific for acetylated-Lysine. In apoptosis, histone H4 was hypoacethlated, but TSA treatment prevented H4 deacetylation, inducing hyperacetylation of all histones.

B. Western blot with R10 antibody (specific for the acetylated forms of H4). Protein loading was checked by Coomassie staining of identical quantities of extracts.

3.13 Biological meaning of the capture of HMGB1 by apoptotic chromatin

The experiments described above showed that HMGB1 has opposite chromatin binding properties in dead cells, depending on the mode of cell death: in apoptotic cells the protein is specifically retained because it binds stably to hypoacetylated chromatin, whereas in necrotic cells HMGB1 is not associated with DNA and is released in the extracellular medium together with other cellular constituents. I tried to clarify the biological meaning of this peculiar behaviour.

3.13.1 Different consequences of cell death: apoptosis versus necrosis

In living organisms, cells die as a part of differentiation and selection processes, and through mechanisms that provide for normal cellular turnover. In physiological contexts, cells die by apoptosis, which represents an active and programmed death process, controlled by intrinsic cellular mechanisms. In contrast, in case of severe injury, cells undergo necrosis, which instead is a "passive" and unprogrammed death. Under both circumstances, dead cells are rapidly cleared from the body, but through different mechanisms and with different consequences.

From a physiological standpoint, apoptotic cell death is primarily distinct from necrosis because it occurs without eliciting an inflammatory response (Kerr et al., 1972). Necrosis is usually the result of trauma, pathologies, poisoning or infection, all events that require prompt reaction and damage containment and/or repair. The primary damage response in mammals is inflammation. Key players in this process are granulocytes and professional phagocytes (macrophages/monocytes and dendritic cells), which are activated by cellular components released by necrotic cells, and produce in response proinflammatory cytokines (Fadok et al., 2001). On the contrary, apoptotic

cells need just to be removed and it is essential that no inflammatory response is mounted. In this process as well, phagocytes have a critical role: macrophages and dendritic cells recognise and engulf apoptotic cells, without secreting any proinflammatory cytokines, and rather produce anti-inflammatory mediators.

Phagocytes discriminate innately between cells that have undergone a physiological death and those that have suffered a pathological death (Cocco and Ucker, 2001). This observation has led to the hypothesis that properties unique to the dying cell must determine the mode and outcome of phagocytic clearance.

3.13.2 HMGB1 released by necrotic cells stimulates proinflammatory cells *in vitro*

The binding behaviour of HMGB1 clearly distinguishes necrotic and apoptotic cells, and might be a good cue to phagocytes, for activating the appropriate responses to unprogrammed and programmed cell death. HMGB1 has already been reported to be a mediator of inflammation (see introduction, paragraph 1.6.1). I then tested directly whether the release of HMGB1 by dead cells can be the immediate trigger for an inflammatory response. Bone marrow cells, containing monocytes in good percentage, were obtained from wild type mice, and stimulated with dead cells from *Hmgb1*$^{-/-}$ or wild type fibroblast lines. As expected, wild type apoptotic cells did not activate monocytes, whereas wild type necrotic cells triggered the production of the proinflammatory cytokine TNF-α, with a typical bell-shaped dose-effect relationship (**Fig. 32**): TNF-α upregulation is always transient upon monocytes activation, and its production rapidly decreases after reaching the peak, in a dose-dependent manner. Significantly, *Hmgb1*$^{-/-}$ necrotic cells were also rather ineffective in eliciting TNF-α

release, indicating that HMGB1 is necessary to promote monocyte activation upon necrosis.

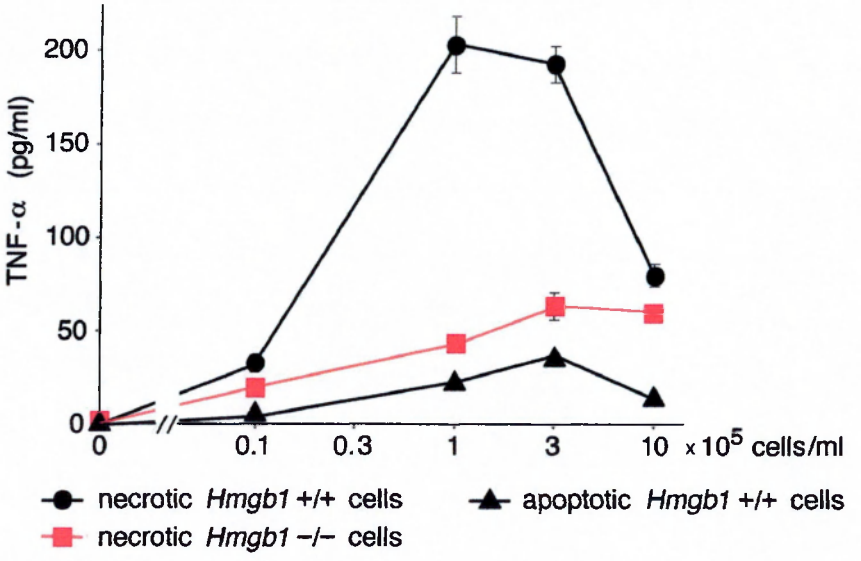

Fig. 32 The release of HMGB1 is necessary to promote inflammatory responses. Necrotic cells lacking HMGB1 do not elicit the production of the proinflammatory TNF-α cytokine by monocytes. Necrotic cells or apoptotic cells were incubated for 18 hours at the indicated final concentration with monocytes from bone marrow, and TNF-α in the supernatant was assayed by ELISA. The experiment was repeated 3 times in triplicate.

3.13.3 HMGB1 released by necrotic cells triggers inflammation *in vivo*

I then tried to confirm the role of HMGB1 release also in an animal model. I could not test whether *Hmgb1*$^{-/-}$ mice have a reduced inflammatory response following tissue necrosis, because these mice survive only a few hours after birth (Calogero et al. 1999). Therefore, I verified whether injections of anti-HMGB1 antibodies can reduce inflammatory responses in wt mice, following liver necrosis.

Acetaminophen (AAP, also known as paracetamol) is a widely used analgesic and antipyretic drug that in toxic doses injures liver in experimental animals and men (Thomas, 1993), by depleting glutathione in hepatocytes and causing oxidant stress. Histological analysis reveals large areas of liver necrosis, concomitant with local inflammation, Kupffer cell activation and the recruitment and sequestration of neutrophils and macrophages into the damaged tissue (Lawson et al., 2000). Levels of liver damage and neutrophil sequestration are strictly proportional until most hepatocytes become necrotic between 12 and 24 hours after AAP poisoning (Lawson et al., 2000).

I administered 300 mg/kg AAP with a single intraperitoneal injection to young mice, and 9 hours later estimated liver injury by measuring alanine transaminase (ALT) activity in serum, and inflammatory cell sequestration by measuring myeloperoxidase (MPO) activity in total liver extracts. One control group of mice (n=8) received no AAP, one group (n=10) received AAP alone, another (n=6) AAP and affinity purified anti-HMGB1 antibodies (300 mg/kg), and the last one (n=8) AAP and irrelevant rabbit antibodies (300 mg/kg). All 3 groups injected with AAP had elevated ALT levels in comparison to sham-treated control, but the differences between the 3 treated groups were not statistically significant. Thus, antibodies do not protect against liver damage, at least at the onset of the inflammatory response. I then used the MPO/ALT ratio to

compare inflammatory cell recruitment, normalized to the level of liver damage (**Fig. 33**). The mice injected with anti-HMGB1 antibodies at the time of AAP poisoning showed a significantly reduced MPO/ALT ratio (1.5± 0.3) both in comparison to mice injected with AAP alone (2.7± 0.3; $p<0.05$), and to mice injected with AAP and preimmune rabbit IgGs (2.4± 0.3; $p<0.05$). Thus, the experiment shows that anti-HMGB1 antibodies are effective in reducing inflammation following liver necrosis. No HMGB1 can derive from activated monocytes and macrophages in our experiment, because HMGB1 secretion from inflammatory cells requires at least 16 hours (Wang et al., 1999; Andersson et al., 2000).

Thus, if HMGB1 released by necrotic hepatocytes is blocked with antibodies, the inflammatory response to necrosis is significantly reduced.

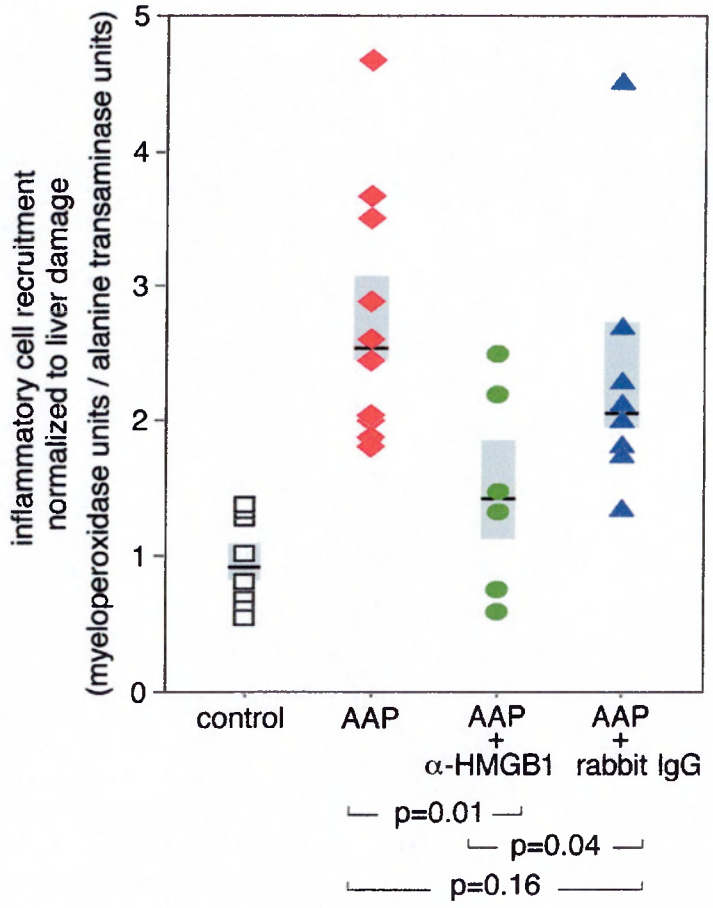

Fig. 33 Anti-HMGB1 antibodies reduce inflammation caused by necrotic hepatocytes. Liver injury was induced in one day old mice by acetaminophen (AAP) overdose. One group of mice was injected intraperitoneally with AAP alone, one with AAP and affinity purified rabbit anti-HMGB1 antibodies, and one with AAP and preimmune rabbit antibodies; control mice received no AAP. After 9 hours, we assessed liver injury by measuring alanine transaminase (ALT) activity in serum, and inflammatory cell recruitment in liver by determining myeloperoxidase (MPO) activity in total liver extracts. The data are expressed here as MPO/ALT ratios, that indicate relative inflammation normalized to liver damage. Each point represents one mouse, the bar indicates the median value, and the grey shade indicates the area included within average $\pm$ standard error. The pairwise comparisons (Mann-Whitney test) between the groups of mice that received anti-HMGB1 antibodies (green ovals), that did not receive antibodies (red diamonds), and that received irrelevant antibodies (violet triangles), is indicated below the graph.

3.13.4 TSA-treated apoptotic cells are proinflammatory

The above experiments prove that HMGB1 represents one of the major diffusible signals of unprogrammed cell death. However, it cannot test whether apoptotic cells escape the inflammatory surveillance because they retain HMGB1. Apoptosed cells start to leak out cellular components only after several hours, and *in vivo* they are generally cleared by phagocytic cells well before this process (termed secondary necrosis) can take place. Therefore, HMGB1 retention might appear irrelevant. However, some apoptotic cells escape prompt clearance, and remain in tissues till later stages. This occurs frequently during development, when many cells synchronously undergo apoptosis, and several unengulfed late apoptotic cells can be easily detectable by TUNEL staining in tissue sections (Potten, 2001; Hanke, 2000; Imamura et al., 2000). Under these circumstances, apoptotic cells probably leak out components but no inflammatory response is induced, suggesting that a safeguard mechanism should exist.

I then directly tested whether cells undergoing post-apoptotic, secondary necrosis would be able to promote inflammatory responses in monocytes. Wild type fibroblasts were treated with TNF-α and incubated for 72 hours, until most LDH was released into the extracellular medium (see Fig. 18). These post-apoptotic cell remnants, which retain HMGB1 bound to chromatin, were unable to promote a strong inflammatory response in monocytes (**Fig. 34**). However, when HMGB1 binding to chromatin was prevented by TSA treatment, secondarily necrotic fibroblasts promoted inflammation as vigorously as primary necrotic cells (killed by freeze-thawing).

From all these data, the retention of HMGB1 by apoptotic cells undergoing secondary necrosis appears to be a safeguard mechanism against the triggering of unwanted phlogystic responses.

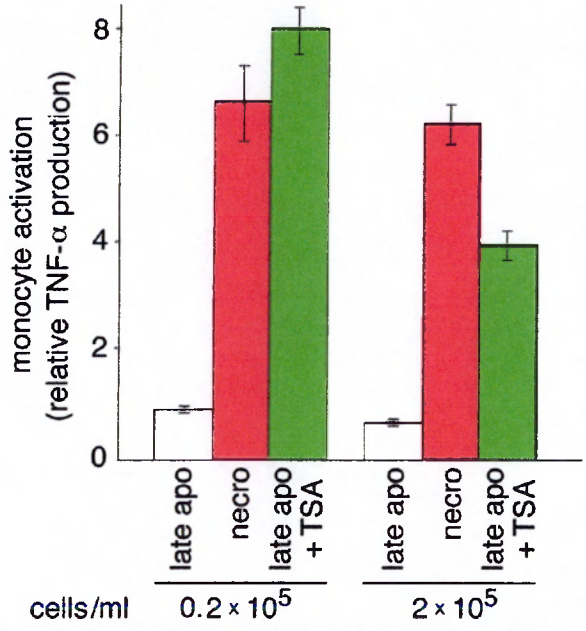

Fig. 34 Apoptotic cells that release HMGB1 are proinflammatory. Apoptotic cells undergoing secondary necrosis and partial autolysis do not promote inflammatory responses, unless HMGB1 is mobilized by treatment with TSA. Apoptosis of 3T3 fibroblasts was induced by TNF-α treatment, in the presence or absence of TSA, for 18 hours at 37°C. Cells were then washed to eliminate TSA, and further incubated for 54 hours before challenging monocytes. TNF-α production after challenge with 0.2 x 10^5 apoptotic cells was considered equal to 1, and the other values were normalized accordingly. The experiment was repeated 3 times in duplicate, with 2 different amounts of apoptotic cells to be sure that TNF-α production by stimulated monocytes would be in the linear range.

4 DISCUSSION

4.1 Transient interaction of HMGB1 with chromatin in living cells

The initial purpose of my thesis work was to characterise the chromatin binding properties of HMGB1, in light of the observation that the protein is only loosely bound to chromatin *in vivo*, despite showing a high affinity to nucleosomes *in vitro*. I generated a GFP-tagged HMGB1 and used it in combination with photobleaching techniques to visualize the dynamics of the protein in living cells. The general indication derived from this analysis is that HMGB1 is extremely mobile. In interphase cells, HMGB1 shows a uniform distribution within the nucleus, which is the result of a continuous and rapid movement throughout the nucleoplasm. The entire pool of HMGB1 roams the nucleus, and less then 1.5 seconds are sufficient for one molecule to traverse the entire compartment, indicating a very high speed.

High mobility is a general feature of nuclear proteins, including structural chromatin proteins, splicing factors, transcription factors and DNA repair enzymes (Phair and Misteli, 2000; Misteli, 2001). This recent finding altered an old view of the nucleus, considered previously as a viscous, gel-like environment full of nucleic acids and proteins. Actually, the volume not accessible to diffusible molecules is less than 15% (Fushimi and Verkman, 1991), and nuclear proteins can move rapidly within the nucleoplasm. Even structural chromatin-binding proteins, thought to be stably bound to DNA, are instead in constant flux on DNA. The movement of most nuclear proteins appears to be independent of energy (in the form of ATP) and likely occurs by a diffusion-based, passive, nondirected mechanism. That is the case for HMGB1 too.

Diffusion is a very effective mode of transport: it is fast, energetically economical and does not need any signal to target the molecule to its site.

HMGB1 appears to be particularly fast, much more than chromatin proteins like HMGN1,2 and histone H1, and transcription factors like NF1. All these proteins have similar size and move by diffusion, thus the difference in the effective mobility measured by FRAP and FLIP must be due to different networks of interactions established by each protein. Generally, incorporation of a protein into a larger complex reduces the protein's apparent mobility, although this has only a small effect unless the complex is very large. A second, more important, reason for slowed protein mobility is the interaction of proteins with relatively immobile nuclear components, like chromatin or the nuclear envelope (**Fig. 35A**). In light of this, the high mobility of HMGB1 is a clear indication of the transient nature of its association to chromatin. Indeed, HMGB1 resides on chromatin at for most 4 seconds before dissociating, suggesting a rapid, saltatory movement between chromatin fibers (**Fig. 35B**). A clear confirmation of this "jumping" mode of binding comes from mitotic cells. In mitosis, HMGB1 is particularly concentrated on chromosomes, but this is not a stable occupancy of chromatin sites. Rather, the protein is continuously exchanged between cytoplasm and chromosomes and the apparently static association is instead the result of a dynamic steady-state. The kinetics of association and-dissociation on DNA are extremely fast, and the transient binding of HMGB1 to chromosomes does not slow significantly its diffusion within the cells.

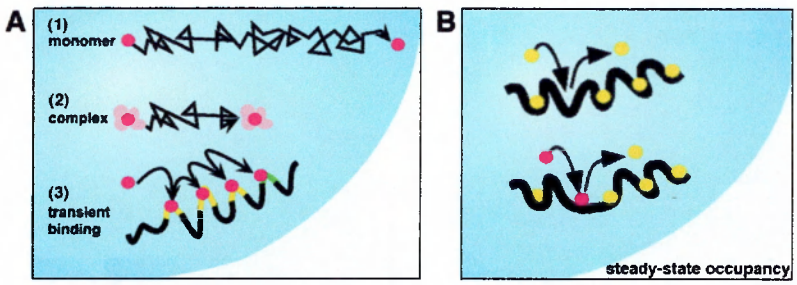

Fig. 35 Apparent mobility of nuclear proteins and steady-state occupancy.

A. The mobility of a protein is determined by its biological properties. Mobility of a monomeric protein is higher than for a complexed protein or for a protein that transiently binds to immobile components in the nucleus such as chromatin. As proteins diffuse through the nuclear space, they are slowed down by their transient interaction with low-affinity binding sites (yellow) before they find a specific, high-affinity site (green).

B. Steady-state occupancy of a chromatin-binding protein is generated by the continuous exchange of proteins from the binding site. Replacement of a chromatin-binding protein (yellow) with a different factor (red) may induce alterations in chromatin structure (Misteli, 2001).

What is the meaning of this hectic movement of HMGB1, on and off DNA? HMGB1 binds DNA in a sequence independent manner and has no specific target. The combination of high mobility and high exchange rate ensures targeting of the protein to its site of action by simple diffusion, during which HMGB1 effectively scans the nucleus for appropriate binding sites. In this view, that HMGB1 encounters a particular protein or a specific binding site on DNA becomes therefore a chance event, and stochastic interactions determine the recruitment of the protein to its binding sites. Before finding a high-affinity binding site, HMGB1 is likely to interact with many low-affinity sites; however, the high content of HMGB1 in the nucleus enhances the probability of productive interactions.

Beyond the fact that this mechanism does not require specific targeting signals, another advantage of such a dynamic and probabilistic behaviour of HMGB1 is the potential to respond promptly to external cues modulating its activity. Many different processes involve HMGB1, some of them simultaneously, others only under specific conditions. If HMGB1 were statically bound to its targets, mechanisms would have to exist to release the protein and make it free for other tasks. The transient nature of its interaction instead ensures a high degree of plasticity: the inherent short residence time on DNA guarantees repeated availability of HMGB1 molecules, and the frequency of the different interactions can be easily shifted depending on the requirement.

As most nuclear proteins appear to be driven by the same stochastic mechanism in their movement, the model might be generalised. Gene transcription, for instance, requires sequential interaction of proteins on promoter regions. The probability of forming a complex is influenced by the availability and residence time on DNA of each assembling component, which, in turn, is determined by its mobility within the nucleus. If a factor is not available within the residence time of a previous intermediate, the assembly process terminates, and transcription does not occur. The Glucocorticoid Receptor (GR), for example, associates only transiently with its target region, residing on DNA only a few seconds (McNally et al., 2000). If no HMGB1 molecule is recruited within the residence time of the GR, the GR dissociates, and the same restriction can be extended to all the other factors involved in the process (**Fig. 36**). Only a fraction of the transcriptional attempts therefore are likely to succeed. Nevertheless, the cooperative binding of many transcription factors enhances the efficiency of transcription apparatus assembly and ensures an adequate rate of transcriptional activation: a single protein molecule with an average speed (diffusion coefficient: 0.2-20 $\mu m^2 sec^{-1}$) and an average

abundance (50,000 copies per cell) encounters a partner molecule of similar abundance every 0.5 seconds, and the formation of preassembled modules in the nucleoplasm dramatically increases the probability of the machinery assembly on DNA.

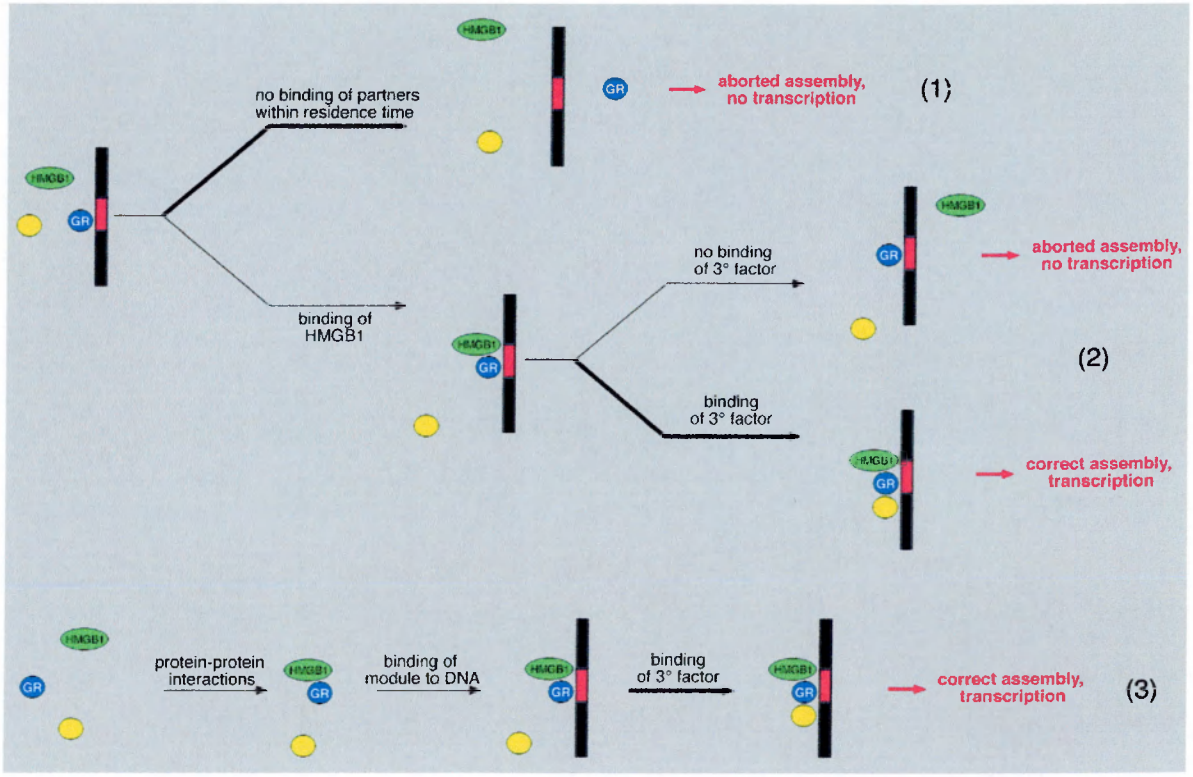

Fig. 36 Stochastic model of gene expression. Assembly of the transcription machinery requires targeting of numerous components to specific DNA sequences. The rate of transcription is determined by the availability of any transcription factor at a locus. The random movement of proteins makes assembly stochastic. A transcription activator, like GR, binds transiently to its target sequence. If no binding partner is recruited within the residence time of the GR, the receptor will dissociate prematurely and no transcription will occur (1). Instead, if a binding partner, like HMGB1, is recruited to the site before the GR dissociates, an intermediate with a longer residence time will form; in this case, the probability to recruit a third factor (yellow) in time is greater and a correct assembly of the machinery may be achieved. The dissociation of the bound dimer however is still possible (2). The efficiency of the assembly is usually enhanced by protein-protein interactions, which form intermediate already in the nucleoplasm: a few preassembled modules with longer residence time associate to DNA, reducing the assembly steps, and the cooperative interactions of many proteins enhanced the stability of the complex (3).

Beyond the possibility to scan the nucleus for appropriate binding sites, the transient nature of HMGB1 interaction with chromatin might underlie also a structural role, in mediating the dynamics of chromatin itself. When observed by time-lapse microscopy, chromatin does not exhibit any dramatic large-scale movements over observations periods of several hours. However, biochemical evidence suggests that small-scale movements, such as remodelling, of the chromatin fibers occur frequently and at all times *in vivo*. Many ATP-dependent chromatin remodellers have been identified (Workman and Kingston, 1998), which mediate relocalisation of intact histones octamers on DNA, in a process termed "nucleosome sliding". The molecular mechanism underlying nucleosome movement is unclear, and several models have been proposed. According to one of the most supported models, the "bulge" model (Havas et al., 2000), during nucleosome sliding, only a small number of DNA-histone bonds are broken at any given time, and very short regions of DNA detach from histone octamer, forming a "bulge". Migration of this "bulge" over the surface of the histone octamer might lead to a change in the translational position of DNA relative to the histones. How nucleosome remodelling machines might facilitate nucleosome mobility through the bulging mechanism is not completely understood.

The high affinity for nucleosomes shown by HMGB1 *in vitro*, and the ability to recognise structured DNA, have suggested the possibility that HMGB1 may be involved in the mechanism of nucleosome sliding. My colleague Tiziana Bonaldi has recently substantiated this hypothesis, showing that HMGB1 interacts with one of the remodelling activities, the ACF complex (ATP dependent Chromatin assembly and remodelling Factor) (Ito et al., 1997), and facilitates ACF-mediated chromatin remodelling (Bonaldi, unpublished). In the presence of HMGB1, both the overall yield of the nucleosome transition and the kinetics of the process are enhanced. The model

emerging from these data suggests that HMGB1 might catalyse nucleosome sliding by binding to the bulge and maintaining it during its movement. This function implicates a continuous association/dissociation of HMGB1 from DNA, following the single nucleosome transitions, whose duration is in the range of milliseconds (**Fig. 37**).

In light of these observations, a general structural role can actually be assigned to HMGB1, even though with a new meaning: HMGB1 is not a static component of chromatin, involved in packaging of bulk DNA; rather it is a dynamic factor which promotes chromatin conformational changes and confers dynamism to the whole structure.

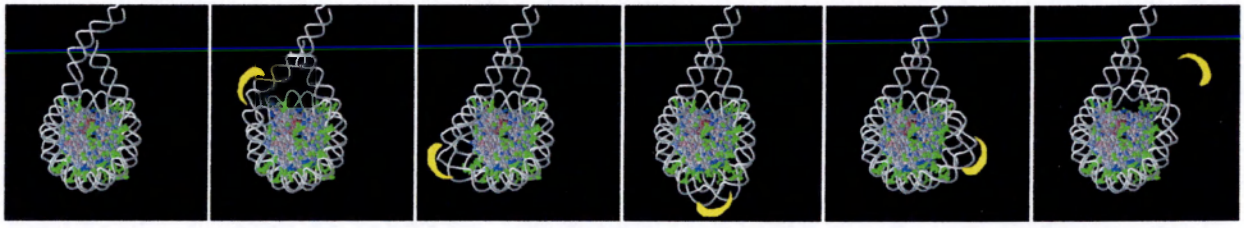

Fig. 37 Bulge model of nucleosome sliding and putative role of HMGB1. HMGB1 (yellow) might facilitate nucleosome sliding by binding the bulge, and accompanying its movement with respect to the core histones. According to this model, HMGB1 would dissociate from DNA after each nucleosome transition, establishing fleeting but repetitive interactions.

4.2 Changes in the chromatin binding properties of HMGB1 during apoptosis

The dynamic behaviour of HMGB1 changes completely when cells undergo apoptosis, and its movement within the cell is blocked. The interactions established with chromatin, which in living cells are transient, in apoptotic cells are extremely stable. This phenomenon appears to be specific for HMGB1, because other proteins do not show any reduction in their mobility during apoptosis, indicating that condensation of apoptotic chromatin does not impair protein mobility in general. The differential behaviour of HMGB1 in living and apoptotic cells is likely not due to changes in post-translational modification of the protein. Rather, the causes of HMGB1 binding to chromatin are chemical and structural alterations in the chromatin that create stable binding sites for HMGB1. In particular, histone H4 is deacetylated during apoptosis, and the inhibition of this deacetylation by trichostatin A treatment completely abolishes HMGB1 binding to chromatin.

The high affinity of HMGB1 to hypoacetylated chromatin might be explained in two different ways. One possibility is that HMGB1 establishes direct contacts with histone H4 and the deacetylated form of H4 is a better partner for HMGB1. However, so far, no clear proof of a direct protein-protein interaction has been obtained for the two molecules. Moreover, by footprinting analysis it has been shown that the preferential binding site for HMGB1 in the nucleosome is the linker DNA (Bonaldi et al., unpublished), and the most important component in determining the stability of the complex appears to be direct interaction with DNA. In light of these observations, we cannot rule out the possibility that a physical interaction with the H4 hypoacetylated tail might increase the affinity of HMGB1 to chromatin in apoptosis; however, it appears unlikely that this is the major factor involved.

The second, more likely, possibility is that the deacetylation of histone H4 determines structural changes in chromatin architecture, which generate stable binding sites for HMGB1. Deacetylation of histone tails is generally associated with chromatin condensation (Davie and Chadee, 1998), and this is the case for apoptosis too. The removal of an acetyl group increases the number of positive charges per nucleosome; this increase, in turn, is likely to cause additional base pairs of DNA to interact with histone cores, thus forcing the central part of the linker to wind in a compensating right-handed toroidal supercoil. In support to this model, electron microscopy studies have shown that the basic conformational feature of apoptotic chromatin is the tight face-to-face packaging of nucleosomes, which might be a direct consequence of increased topological constraints of the linker DNA (**Fig. 38**) (Allera et al., 1997). In any event, a stressed DNA is an excellent substrate for HMGB1, and a strong binding of the protein to the supercoiled DNA linker might be a plausible explanation for its stable binding to apoptotic chromatin.

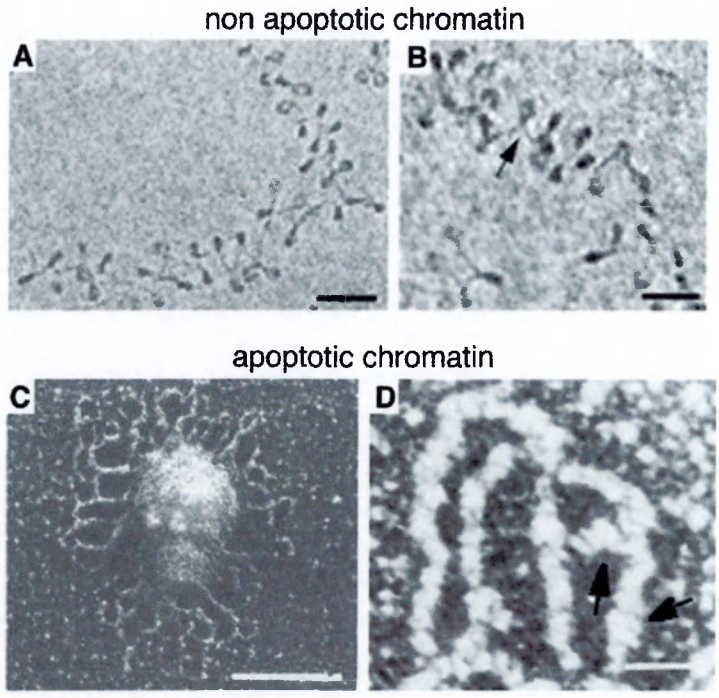

Fig. 38 Differences between "living" and apoptotic chromatin fibers.

A-B. Electron micrographs of segments of unfixed, unstained chromatin fibers released from nuclei of living chicken erythrocytes into low-salt buffer and observed in the frozen hydrated state. Within the fibers, individual nucleosomes are seen in a variety of orientations and appear as pear-shaped structures connected by linker DNA (arrow in B) that form "stems" at the entry-exit sites (Bednar et al., 1998). Scale bars, 30 nm.

C-D. Electron micrographs (C: low magnification, scale bar, 0.2 μm, D: higher magnification, scale bar, 0.03 μm) of chromatin isolated at low ionic strength from nuclei of apoptotic thymocytes. The arrows in D indicate short regions of the 11-nm filament where core particles are sharply imaged. Nucleosomes are tightly associated face-to-face, and linker DNA, invisible, is likely to stick out the nucleosomes array, assuming a stressed confirmation (Allera et al., 1997).

4.3 HMGB1 as a signal of unprogrammed cell death.

I have shown that the binding of HMGB1 to chromatin depends on the viability of the cell. In living cells, the protein is highly dynamic in its binding to both condensed and decondensed chromatin. In dying cells, however, this rapid turnover is frozen: in apoptotic cells HMGB1 is specifically retained by binding stably to hypoacetylated chromatin, whereas necrotic cells leak out the protein, together with other cellular constituents. The release of HMGB1 by necrotic cells is actually a direct consequence of its transient association with chromatin in living cells. The sudden breakage of the membrane, which renders necrotic cells the physiological counterpart of permeabilised living cells, causes HMGB1 to dilute in the extracellular milieu until all the molecules dissociating from chromatin diffuse out. This is therefore a totally passive release of the protein.

The opposite behaviour of HMGB1 in necrosis and apoptosis make it a good clue for proinflammatory cells to distinguish between programmed and unprogrammed cell death. It is known since Celsius (100 A.D.) that the four cardinal signs of inflammation, *rubor* (redness), *calor* (heat), *tumor* (swelling) and *dolor* (pain), always accompany tissue injury, as consequences of increased blood flow to the affected area. Nevertheless, despite the well-known correlation between necrosis and inflammation, the molecular basis of recognition and reaction to necrotic cells is still unknown. A variety of molecules released by lysed cells have been implicated as putative recognition elements, including Heat Shock Proteins, ATP and extracellular matrix breakdown products (Sauter et al., 2000; Schnurr et al., 2000; Wrenshall et al., 1999), but the picture is still blurred and no definitive assignment has been made.

I found that necrotic cells lacking HMGB1 are poorly inflammatory, compared to wt cells, indicating that HMGB1 is one of the most important signals mediating the

phlogystic response to necrosis. The response is not totally abolished in the absence of HMGB1, indicating that it is not the only factor involved in the process. One possibility is that HMGB2, which is released by lysed cells (Ronfani, unpublished), might cooperate with HMGB1 in activating proinflammatory cells and even in the absence of HMGB1 might determine a minimal response. At the moment, we do not know whether HMGB2 has a cytokine activity as well, but considering the high similarity of the two proteins, this is likely. Alternatively, other factors, unrelated to HMGB1, might indeed have similar functions in inflammation.

In activating proinflammatory cells, HMGB1 behaves as one of the long-sought "danger signals" to the immune system, as initially proposed in theories of tolerance and immunity. The concept of a danger signal was introduced in 1994 as part of a model of immunity that suggests that the primary driving force of the immune system is not the distinction between self and non-self, but the need to detect and protect against danger, both exogenous and endogenous (Matzinger, 1994). In this view, bacteria activate the innate immune system because endotoxins, recognised by macrophages and monocytes, signal the *exogenous* danger. In the same way, necrotic cells stimulate proinflammatory cells because released HMGB1 is recognised as signal of *endogenous* danger. The signal recognising receptors on professional phagocytes act therefore as sentinels of the immune system, which detect hazardous situations.

In the hypothesis that the *passive* release of HMGB1 by necrotic cells can be used as a simple mechanism of natural immunity, it can be speculated that monocytes/macrophages have evolved the ability to *actively* release the danger signal without dying. They secrete HMGB1 in response to other stimuli, for example endotoxins or proinflammatory cytokines, using it as a late mediator of inflammation in case of exogenous danger. In addition, they might also provide an amplification step of

the HMGB1 signal, which acts as an early mediator of the response in case of endogenous danger. In this way, even a small amount of HMGB1 released by a damaged cell would be able to trigger a robust reaction.

In principle, any substance that is normally found inside the cell and that can be released upon damage to the plasma membrane, could act as a danger signal; why then has HMGB1 been "chosen" as the primary message of death? High abundance and ubiquitous presence are the first requirements for a robust cue; essentially every cell in an organism contains HMGB1, and about one million molecules of the protein are released per cell in the case of necrosis. Core histones, though ubiquitous and even more abundant, cannot be signals of damage, as they remain anchored to the insoluble chromatin of necrotic cells and are not diffusible. Moreover, a good danger factor must be able to differentiate unprogrammed, proinflammatory, cell death from programmed, non-inflammatory, death, and HMGB1 has indeed opposite behaviours in necrotic and apoptotic cells: it is released by necrotic cells, triggering inflammation, whereas it is retained by apoptotic cells, tightly bound to chromatin, averting inflammation.

An important point however must be underlined in this context: under normal circumstances, apoptotic cells do not release anything at all, because they are rapidly cleared by phagocytic cells. Several "eat me" signals are present on the surface of apoptotic cells, mediating recognition and uptake by professional phagocytes. One of the earlier events occurring to apoptosing cells is the exposure on the outer-membrane leaflet of phosphatidylserine, a lipid normally located to the inner leaflet of the plasma membrane. As soon as the lipid is exposed, macrophages recognise it through a specific receptor, and engulf the dying cells before they lyse (Fadok et al., 2000). In light of that, even if HMGB1 were not sequestered by binding to chromatin, in most cases apoptotic cells would not trigger inflammation anyway, because they wouldn't have time to lyse

and release HMGB1. Thus, HMGB1 retention does not appear to be the major mechanism to prevent inflammation. Nonetheless, the clearance of apoptotic bodies is not totally efficient and some cells routinely escape rapid removal, undergoing secondary necrosis. In that case the paralysis of HMGB1 is necessary. I showed that late apoptotic cells, although permeable to soluble molecules, indeed are not as inflammatory as primary necrotic cells. Moreover, in further support of our idea, if HMGB1 is detached from chromatin by TSA treatment, even late apoptotic cells become proinflammatory. The real difference therefore is not between necrotic and apoptotic cells, but between primary necrotic and secondarily necrotic, late apoptotic cells. Uncleared apoptotic cells would be probably much more dangerous than they actually are, if HMGB1 were not retained by chromatin. Thus, the capture of HMGB1 represents a safeguard mechanism in case of non-rapid clearance of apoptotic bodies.

4.3.1 Various responses to the traumatic demise of nearby cells.

Proinflammatory cells of the innate immune system are not the only targets of released HMGB1, and "chromatin spillage" could be a more general message of death, read by all the neighbours of traumatically dead cells.

In collaboration with Angelo Manfredi in our institute, we found that HMGB1 can also promote dendritic cell maturation. Dendritic cells (DCs) are professional antigen presenting cells which take up a diverse array of antigens and present them to T cells, initiating the adaptive immune response (Gallucci and Matzinger, 2001). DCs exist in two functionally and phenotypically distinct states, immature and mature. Immature cells are adept at endocytosis but express relatively low levels of costimulatory molecules for T cell activation. Thus, immature DCs, which are mostly present in peripheral tissue, can take up antigens but do not present them efficiently to T cells.

After detecting a danger signal, immature DCs transform into mature DCs, cells with a reduced capacity for antigen uptake but now with an exceptional capacity for T cell stimulation. This transition is accompanied by a dramatic cytoplasmic reorganization and migration to lymphoid organs, where T cell priming takes place. Necrotic cells can activate DCs, and HMGB1 was found to act as a danger signal also in this context. Purified HMGB1 can induce the maturation of human dendritic cells *in vitro*, inducing upregulation of different markers, including MHC class I and II, with an efficiency at least comparable to LPS or TNF-α. The morphological changes associated with dendritic cell maturation are also seen, and HMGB1-challenged DCs are capable of priming T cells. In addition, *in vivo* HMGB1 favours the activation of antigen presenting cells and the initiation of immune responses, transforming poorly immunogenic apoptotic lymphoma cells in efficient vaccines (Rovere et al. unpublished). Thus, the release of HMGB1 by damaged cells not only induces inflammatory responses by macrophages, but also represents a signal to the adaptive immune system, which can regulate the balance between immunity and tolerance towards intracellular antigens.

Cells not related to the immune system as well can detect the message of death. In addition to inflammation, tissue damage promotes tissue repair and remodelling processes. We have recently shown that HMGB1 elicits migratory responses in vascular smooth muscle cells. In case of damage of the endothelium, injured cells release their HMGB1 both in the bloodstream (possibly causing inflammation of nearby endothelia) and towards the intimal layer of the vessel, causing migration and proliferation of smooth muscle cells (Degryse et al., 2001). In physiological circumstances this contributes to repair the damage. However, this process may also have pathological consequences: necrotic cells accumulate in later stages of atherosclerosis, and by

releasing their HMGB1, they could support a continuous process of inflammation, and stimulate smooth muscle cell migration, leading to intima thickening and stenosis. More generally, metalloproteinases (MMP), which play an essential role in tissue repair facilitating cell migration, are rapidly induced after tissue injury (Li et al., 2001). HMGB1 has been shown to activate MMPs, inducing degradation of the extracellular matrix, and thereby promoting wound healing responses to tissue damage (Taguchi et al., 2000).

We can also speculate that cancer metastasis (Taguchi et al., 2000) could be caused by the release of HMGB1 from necrotic cells in the interior of large tumour masses. Released HMGB1 could be recognised by cancer cells at the periphery of the tumor mass, stimulating them to execute dispersal programs.

Finally, also stem cells might use HMGB1 to detect dead cells, and start to divide replacing the damaged tissue. Experiments are in progress to see whether injection of HMGB1 in mouse muscles can mimic tissue damage and activate satellite stem cells.

The main issue remaining outstanding is the identity of the receptor which reads out the danger signal. To date, the only high-affinity receptor for HMGB1 is RAGE, which is expressed on a wide range of cellular types, and thus is a good candidate. It has been shown that RAGE mediates HMGB1 signalling in the migration of vascular cells and tumor invasion (Degryse et al., 2001; Taguchi et al., 2000). Also macrophages/monocytes express RAGE, and RAGE has been already involved in inflammation, as receptor for calgranulin (Hofmann et al., 1999). Nevertheless, a considerable body of evidence derived from immunological studies on necrotic cells suggests that another receptor could be involved in the process. The Toll-like receptor (TLR) family represents a set of at least nine surface molecules essential for recognition of microbe-derived products (Akira et al., 2001). Different TLRs are responsible for

detection of LPS, bacterial DNA, yeast lipopeptides, and through a common pathway, which ultimately involves NF-kB, activate host innate immunity against exogenous danger. The reaction to endogenous danger, which occurs with very similar formalities, might use the same "sentinels". Indeed, necrotic cells have been identified as potent NF-kB inducers, and this activation requires signalling molecules of the TLR pathway (Li et al., 2001). Thus, Toll-like receptors could be responsible for mediating HMGB1 signalling in the inflammatory response.

In conclusion, in light of these data HMGB1 puts itself forward as a general danger signal that informs cells of the traumatic, non-programmed death of their neighbours. This function of HMGB1 depends on its intrinsic properties of abundance, rapid and reversible association with chromatin in living cells, and stickiness to apoptotic chromatin.

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