CHROMATIN CONDENSATION AND FRAGMENTATION CAUSED BY CUG-INITIATED FGF-2 IN CARDIOMYOCYTES

BY

GUANGPING SUN

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ABSTRACT

Fibroblast growth factor-2 (FGF-2) is a multifunctional mitogenic factor which is found in 21-25 kDa high molecular weight (HMW), or 16-18 kDa low molecular weight (LMW) forms, resulting from translation initiation from unconventional CUG- or conventional AUG- start sites, respectively. Previous studies have demonstrated that rat HMW- but not LMW-FGF-2, introduced into cardiac myocytes by gene transfer, caused a distinct nuclear phenotype, characterized by condensed chromatin and formation of several DNA 'clumps' inside the nucleus. In the present study we investigated first whether human HMW FGF-2, sharing 82% homology with its rat counterpart at the CUG-initiated extension, was also capable of eliciting the same phenotype as rat HMW FGF-2. Secondly, we investigated whether the mechanism of chromatin condensation and separation caused by HMW FGF-2 engaged an apoptosis-like mechanism. Finally, we examined whether the HMW FGF-2 induced nuclear phenotype resulted from a mitosis-like mechanism. Using cDNAs coding for HMW- or LMW- human FGF-2 and transient gene transfer of neonatal cardiomyocytes we established that the human versions of FGF-2 had similar properties to their rat counterparts. Human HMW FGF-2 localized predominantly to the nucleus and induced chromatin condensation and separation in over 20% of overexpressing cells. Human LMW FGF-2 localized to the nucleus and cytosol, and was not associated with chromatin alterations. The nuclear phenotype caused by HMW FGF-2 does not appear to be related to apoptosis based on the following findings: (a). The nuclei in HMW FGF-2 overexpressing cells were TUNEL negative, and their nuclear lamina, assessed by anti-lamin B immunostaining, appeared intact. (b). We did not detect a DNA ladder. (c). A number of manipulations that have been shown to

prevent apoptosis in several systems, including overexpression of Bcl-2, inhibition of ICE-proteases, and overexpression of LMW FGF-2 did not prevent the effect of HMW FGF-2. The nuclear phenotype caused by HMW FGF-2 does not appear to be related to mitosis: The nuclei in HMW FGF-2 overexpressing myocytes did not stain for the phosphorylated forms of histone H1, or H3. Phosphorylation of H1, H3 is associated with chromatin condensation and chromosome separation observed in mitosis. In conclusion, we have provided evidence that the nuclear phenotype caused by overexpression of HMW FGF-2 in cardiac myocytes likely represents a novel mechanism of nuclear disruption. The physiological significance of this observation remains to be established.

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LIST OF ABBREVIATIONS

μg	microgram
μl	Microliter
Bcl-2	B cell leukemia/lymphoma-2
CAT	chloramphenicol acetyltrasferase
cDNA	complementary deoxyribonucleic acid
CMF-PBS	Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline
DMEM	dulbecco's modified Eagle's medium
Egr-1	early growth response factor-1
ER	endoplasmic reticulum
FBS	fetal bovine serum
h	hour
HBS	Hepes-buffered saline
HMW FGF-2	High molecular weight FGF-2
HS	horse serum
ICE	interleukin-1 beta-converting enzyme
kDa	kilodalton
LMW FGF-2	Low molecular weight FGF-2
MAP kinase	mitogen-activated protein kinase
min	minute
ml	milliliter
°C	degree centigrade

PARP	poly(ADP-ribose) polymerase
PT	permeability transition
SMC	structural maintenance of chromosomes
TUNEL	TdT-mediated dUTP nick end labeling
Z-DEVD-fmk	benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone
Z-VAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

INTRODUCTION

1 General Introduction

Basic fibroblast growth factor (bFGF), also called FGF-2, is a member of a large family of heparin-binding proteins, which consist of at least 19 structurally related polypeptide growth factors (Nishimura et al. 1999). FGF-2 exists in all tissues examined so far and has several different effects in different cell and organ systems. It affects cell proliferation, cell migration, embryonic development, cell differentiation, survival, tumor angiogenesis, and malignant transformation (Bikfalvi et al. 1997; Kardami et al. 1995).

The FGF-2 gene is located on chromosome 4q26-27 (Emoto et al. 1997). FGF-2 genes have been cloned from human, opossum, bovine, rat, chick, mouse, sheep, Xenopus, and newt (Szebenyi and Fallon 1999).

Human FGF-2 exists in four different molecular weight isoforms that originate from alternative translation initiation codons within the same mRNA molecule. Low molecular weight (LMW) FGF-2 (18-kDa) initiates at an internal methionine (AUG) codon, while the three high molecular weight (HMW) forms (22, 22.5, and 24 kDa) initiate at three leucine (CUG) codons located C-terminal to the AUG. Therefore, in addition to a varying length of N-terminal extension, the three HMW FGF-2 contain the complete LMW FGF-2 sequence at their C termini (Florkiewicz and Sommer 1989; Prats et al. 1989). Rat and chick FGF-2 also exist in CUG- and AUG-initiated species (Szebenyi and Fallon 1999).

There are three coding exons in FGF-2 (Sutherland et al. 1996; Burdine et al. 1997). Each of the exons encodes parallel β strands, which fold into a distinct structural domain.

FGF-2 is found both outside and inside of the cells (Mignatti et al. 1992). The mechanism for release of FGF-2 from cells is unclear because FGF-2 does not contain a signal sequence (Abraham et al. 1986). FGF-2 can be released from cells after cell death, wounding, or chemical injury (McNeil et al. 1989; Muthukrishnan et al. 1991), or irradiation (Haimovitz-Friedman et al. 1991).

However, healthy cells also release FGF-2. FGF-2 neutralizing antibodies inhibit the migration of an isolated single cell expressing FGF-2 (Mignatti et al. 1991). The drugs that inhibit the classic ER-Golgi secretion pathway do not inhibit FGF-2 dependent cell migration (Mignatti et al. 1991). Therefore a nonclassic release pathway other than the classic secretion pathway, cell death, or wounding can lead to the export of FGF-2 (Mignatti et al. 1991).

Some of the characteristics of the mechanism for FGF-2 secretion are beginning to emerge. Florkiewicz and colleagues (Florkiewicz et al. 1998) provided some evidence that the export of FGF-2 may involve interaction with the plasma membrane ion pump Na^+, K^+-ATPase. Piotrowicz et al. (Piotrowicz et al. 1997) furthermore have shown that the 27 kDa heat shock protein increases the rate of secretion of FGF-2 (especially the HMW form) from bovine arterial endothelial cells.

2 HMW FGF-2 versus LMW FGF-2

2.1 Distribution & Localization

Our laboratory has demonstrated that immature cardiac ventricles accumulate predominately the HMW FGF-2 while the 18 kDa form is prevalent in the adult myocardium (Liu et al. 1993). Furthermore, relative levels of HMW FGF-2 differ

between different tissues, being highest in the adult brain and spleen and lowest in striated muscle (Liu et al. 1993). Hypothyroidism increased the relative levels of HMW FGF-2 in the heart but not other tissues (Liu et al. 1993).

Similar findings were subsequently obtained by other investigators: Studies with transgenic mice overexpressing human FGF-2 showed that some tissues preferentially accumulate HMW FGF-2, while other tissues have predominantly 18 kDa FGF-2; the tissue specific expression of FGF-2 isoforms appears to be translationally regulated (Coffin et al. 1995). Several cis-regulatory sequences have been found that contribute to the translational control of FGF-2 mRNA (Prats et al. 1992). In particular, an IRES (internal ribosome entry site) has been found in the FGF-2 mRNA leader (Vagner et al. 1995). This allows FGF-2 mRNA translation to occur via a new mechanism different from the classical cap-dependent scanning mechanism (Kozak 1989).

HMW isoforms of FGF-2 are localized in the nucleus, whereas 18-kDa FGF-2 is found predominantly in the cytosol and nucleus (Quarto et al. 1991; Renko et al. 1990; Bugler et al. 1991; Florkiewicz et al. 1991; Pasumarthi et al. 1994). 18-kDa FGF-2 can also be exported to the cell surface, where it is localized to the basement membrane or extracellular matrix in association with matrix heparins and heparan sulfate proteoglycan (Moscatelli et al. 1991; Miao et al. 1996). Nuclear distribution of 18 kDa FGF-2 was seen in some situations, and may occur via diffusion of 18 kDa FGF-2 into the nucleus through the nuclear membrane or it may enter after the nuclear membrane is disrupted during the mitotic cycle (Silver 1991).

The intracellular distribution of the HMW FGF-2 is determined by the N-terminal extensions, not found in 18 kDa FGF-2, which act as nuclear localization signals.

Chimerical proteins consisting of the N-terminal domain of HMW FGF-2 joined to proteins that normally localize in the cytoplasm can be targeted to the nucleus (Quarto et al. 1991; Bugler et al. 1991). The nuclear targeting sequence of SV-40 T-antigen joined to 18 kDa FGF-2 does not allow nuclear accumulation of the fusion protein. This indicates that 18 kDa FGF-2 contains an inhibitory sequence for nuclear transport, which can be overcome by presence of the N-terminal extension (Florkiewicz et al. 1991).

Posttranslational methylation of arginine residues may play a critical role in nuclear localization of HMW FGF-2. The amino-terminal extension of HMW FGF-2 contains 55 amino acids and has eight potential sites for arginine methylation (Najbauer et al. 1993). The translocation of newly synthesized HMW FGF-2 into the nucleus appears to require the posttranslational methylation, since a methytransferase inhibitor markedly reduces the nuclear accumulation of endogenous HMW FGF-2 (Pintucci et al. 1996).

2.2 Regulation of Expression

2.2.1 At the Transcriptional Level

Both rat and human FGF-2 promoters lack typical TATA or CCAAT box, but have GC-rich regions (Shibata et al. 1991). Transcription factors Sp-1 or early growth response factor-1 (Egr-1) may be involved in the activation of transcription, since binding sites for these proteins are contained in the GC-rich region, which is associated with transcription initiation (Pasumarthi et al. 1997; Biesiada et al. 1996). The expression of Egr-1 transcription factor after injury is triggered by release of FGF-2 and dependent on paracrine action of FGF-2 (Santiago et al. 1999). FGF-2 can stimulate its own expression

through an autoregulated transcriptional response that requires the Egr-1; Egr-1 binds to two DNA elements in the FGF-2 promoter and positively regulates its transcription (Wang et al. 1997). The transcriptional induction of Egr-1 is mediated through a mitogen-activated protein kinase (MAP kinase) related mechanism (Biesiada et al. 1996; Schwachtgen et al. 1998; Santiago et al. 1999).

α-Adrenergic stimulation (norepinephrine, or phenylephrine) increases local FGF-2 synthesis at the transcriptonal level in cardiac myocytes, this increase is believed to be involved in the response of cardiomyocytes to injury as well as the maintenance of the healthy myocardium (Detillieux et al. 1999).

Many factors stimulate the expression of FGF-2, such as glucocorticoids, cAMP and forskolin (Meisinger et al. 1996; Riva et al. 1996; Halaban 1996). P53 may also regulate FGF-2 expression. When p53 and FGF-2 cDNAs were co-transfected into human glioblastoma and hepatocellular carcinoma, expression of endogenous FGF-2 was increased by mutant type p53; the FGF-2 gene promoter was responsive to p53. Wild-type p53 represses FGF-2 gene expression; mutant p53 enhances FGF-2 expression (Ueba et al. 1994).

2.2.2 At the Translational Level

The expression of the CUG-initiated forms of FGF-2 is translationally down regulated when cell density increases in normal human skin fibroblast cultures (Galy et al. 1999). In contrast, the CUG-initiated FGF-2 expression is constitutive in cancerous SK-HEP-1 cells as well as in human skin fibroblasts transformed by SV40 large T antigen (Galy et al. 1999). The density-dependent regulation of FGF-2 expression in

normal cells employs a cap-dependent scanning mechanism, whereas the constitutive expression of FGF-2 in transformed cells and cancer cells involve an IRES-dependent translational regulation mechanism. Initiation at the AUG codon in low-density normal human skin fibroblast occurs via an internal ribosome entry mechanism, and there is a second IRES in the FGF-2 mRNA which would drive the AUG-initiated form expression (Galy et al. 1999).

In general, the CUG-initiated HMW FGF-2 isoforms predominate in transformed cell lines; whereas the AUG-initiated 18-kDa form is more prevalent in normal cells. Constitutive expression of the AUG-initiated form leads to transformation of adult bovine aortic endothelial and NIH 3T3 cells, whereas expression of the CUG-initiated forms lead to immortalization of adult bovine aortic endothelial cells and confers a unique phenotype to NIH 3T3 cells (Couderc et al. 1991; Quarto et al. 1991). The AUG-initiated FGF-2 expression is approximately related to the mRNA levels in the different cell types, but there does not seem to be a correlation between FGF-2 mRNA and expression of the CUG-initiated FGF-2 (Vagner et al. 1996).

CUG-initiated FGF-2 can be induced by stress (heat shock treatment which can abolish the cap-dependent translation). This effect is time dependent, and correlates with the change in FGF-2 mRNA levels (Piotrowicz et al. 1997).

The 5'region of FGF-2 mRNA contains the cis-acting elements which are necessary for the cell-type specific control of alternative translation initiation, presumably through binding of several cell proteins to this leader region. So the CUG-initiated forms are translationally regulated by trans-acting factors that would be active in transformed cells but only activated as a response to stress in normal cells (Vagner et al.

1996).

The expression of high molecular weight (HMW)-isoforms (22 and 24-kD isoforms) but not 18-kD isoform was selectively increased by cytokines in cultured rat astrocytes (Kamiguchi et al. 1996).

2.3 Biological Activity

There exist both differences and similarities in the biological activity of CUG- versus AUG-initiated FGF-2. Both appear to bind to the plasma membrane receptors with similar properties, and to have similar receptor-mediated mitogenic activity (Patry et al. 1994). On the other hand, when the different forms are expressed in a fibroblast cell line, 3T3 cells, while both are mitogenic, they have different effects on cell migration, or integrin expression: LMW, but not HMW, FGF-2 stimulates migration and integrin expression (Piotrowicz et al. 1999).

Bikfalvi et al. found that (a). NIH 3T3 cells expressing 18-kDa FGF-2 have high motility and surface-associated 18 kDa FGF-2. In contrast, cells expressing HMW FGF-2 have low motility and virtually no surface-associated FGF-2. (b). FGF-2 receptor numbers are decreased in cells expressing 18-kDa FGF-2, but do not change in cells expressing HMW FGF-2. (c). Expression of a dominant negative FGF-2 receptor inhibits the migration and suppresses the growth in soft agar of cells expressing 18-kDa FGF-2, but has no effect on the growth of cells expressing HMW FGF-2. These findings demonstrate that the AUG-initiated FGF-2 acts through an autocrine or paracrine pathway, while the CUG-initiated isoforms of FGF-2 act through an intracrine pathway (Bikfalvi et al. 1995).

In cardiac myocytes, overexpression of both HMW and LMW FGF-2 increases cardiomyocyte number, DNA synthesis, and mitotic index by a receptor-dependent mechanism. The overexpression of HMW FGF-2, but not LMW FGF-2, also increased binucleation and induced DNA clumping in cardiac myocytes by a receptor-independent mechanism, suggesting these effects are through an intracrine pathway (Pasumarthi et al. 1996).

LMW FGF-2 has been demonstrated to prevent apoptosis induced by different triggers, in several systems, including endothelial cells (Karsan et al. 1997), bladder cell lines (Miyake et al. 1998), glioma cells (Murai et al. 1996), lens epithelial cells (Wang et al. 1999), L929 cells (Gardner and Johnson 1996), HC11 mammary epithelial cells (Merlo et al. 1996) and others. Overall, LMW FGF-2 appears to confer protection from apoptosis in many tissues, as illustrated in recent review (Rosfjord and Dickson 1999), although for most of the earlier studies it is not clear whether protection occurs by preventing apoptosis or necrosis.

Although most of the available evidence points to LMW FGF-2 as preventing apoptosis, Yokoyama et al (Yokoyama et al. 1997) provided some evidence that it may actually trigger the apoptotic process in developing chick retina neural cells in culture. It is therefore possible that the ability of LMW FGF-2 to prevent apoptosis is cell-type or stage- specific. Funato et al (Funato et al. 1997) also reported that LMW FGF-2 enhanced apoptosis in myofibroblastic cells, however their data are very preliminary, without a quantitative assessment of apoptosis, nor a dose-dependent examination of the effect; furthermore their work centers on the effects of added FGF-2 on serum-deprived cells that are already compromised and perhaps sensitized towards apoptosis. It is therefore too

early to decide whether LMW FGF-2-induced apoptosis is a more general phenomenon or restricted to some cell types or conditions.

There are no reports associating HMW FGF-2 with apoptosis.

3 FGF-2 in the Heart

FGF-2 is present in the nucleus, cytoplasm, cell membrane and gap junctions of cardiac myocytes (Pasumarthi et al. 1994; Pasumarthi et al. 1996; Kardami et al. 1991), and plays a vital role in the proliferation and differentiation of these cells (Kardami et al. 1995).

In the postnatal heart, FGF-2 is released on contraction. Electrical or adrenergic stimulation increases the heart rate and force, and therefore increases FGF-2 release (Clarke et al. 1995; Kaye et al. 1996). The mechanism of export involves a transient, non-lethal disruption of the plasma membrane, a phenomenon that occurs in many tissues exposed to high levels of mechanical stress (McNeil and Steinhardt 1997). Since FGF-2 has a negative inotropic effect (Padua et al. 1998), its release may help the heart compensate after excessive adrenergic stimulation. It may also trigger the hypertrophic pathway in vivo.

HMW and LMW FGF-2 species have been identified in the heart. The 18 kDa FGF-2 predominates in the adult rat heart, while HMW FGF-2 predominates in neonatal rat heart (Liu et al. 1993). In gene transfer studies, HMW FGF-2 isoforms initiated from CUG codons of rat FGF-2 mRNA, preferentially localize to the nucleus of embryonic chicken cardiac myocytes and neonatal rat cardiac myocytes, whereas the 18 kDa form resulting from the AUG codon localizes to both the nucleus and the cytoplasm

(Pasumarthi et al. 1994; Pasumarthi et al. 1996). Differential localization implies distinct function for LMW versus HMW FGF-2.

Overexpression of both 22/21.5 and 18 kDa FGF-2 induced a significant increase in cell number, DNA synthesis and mitotic index in neonatal rat cardiac myocytes compared to non-overexpressing cells (Pasumarthi et al. 1996). HMW and LMW FGF-2 stimulated proliferation to a similar degree, and their effects were inhibited by neutralizing antibodies against FGF-2, indicating involvement of an autocrine or paracrine pathway. On the other hand, overexpression of 22/21.5 kDa FGF-2 (but not LMW FGF-2) was associated with a significant increase in binucleation and DNA clumping, which was not blocked by the neutralizing antibodies against FGF-2. These data demonstrate the HMW and LMW FGF-2 can have different biological function, and the HMW FGF-2 has its effects on binucleation and DNA clumping through an intracrine mode (Pasumarthi et al. 1996).

FGF-2 is involved in cardiac response to injury. In an isoproterenol-induced cardiomyocyte injury model, there was a transient increase in HMW FGF-2 24 hours after treatment, coinciding with intense cellular proliferation and infiltration in the area of injury (Padua and Kardami 1993). LMW FGF-2 was also increased, and its increases correlated with the chronic response of the heart to injury. Increased levels of FGF-2 have also been detected in the mdx model of cardiac lesion development (Anderson et al. 1991), and in myocardial infarction (Kardami et al. 1993; Padua et al. 1996). Our laboratory has some evidence that FGF-2 protects cardiac myocytes from H_2O_2-induced apoptosis (Kardami et al. 1993; Padua et al. 1996).

Autocrine FGF-2 signaling has been reported to contribute to smooth muscle cell migration (Sato et al. 1991). FGF-2 may also play a role in cell recognition, adhesion and communication. It has been shown that FGF-2 is localized within the intercalated disc region of cardiomyocytes. Immune electron microscopy showed FGF-2 localized on the cytoplasmic face of gap junctions (Kardami et al. 1991). FGF-2 decreases cell to cell communication by acting on connexin 43 in neonatal rat cardiac myocytes via the phosphorylation of a serine residue on the cytosolic face of gap junctions (Doble et al. 1996).

FGF-2 has cardioprotective effects in rat heart ischemia-reperfusion injury. When FGF-2 was administered to the ischemic heart by perfusion, it was localized to the periphery of cardiomyocytes, and induced a significant recovery of mechanical function (Padua et al. 1995). This cardioprotection was mediated by the activation of PKC at the sarcolemmal sites (Padua et al. 1998).

Thyroid hormone may down-regulate accumulation of the high molecular weight forms of FGF-2 in the heart, because elevated levels of 21.5 to 22 kDa forms of FGF-2 were detected in hypothyroid compared to euthyroid cardiac ventricles (Liu et al. 1993).

4 FGF-2 Signal Transduction

The biological functions of FGF-2 are mediated by its interaction with a dual receptor system consisting of high-affinity tyrosine kinase FGF-receptors (FGFRs) and low-affinity heparan sulfate proteoglycans (Jaye et al. 1992; Klagsbrun and Baird 1991).

The FGFR family consists of at least four members (FGFR1, 2, 3, and 4) (Basilico and Moscatelli 1992; Jaye et al. 1992; Jin et al. 1994; Liu et al. 1995). These

receptors have conserved features including a cytoplasmic tyrosine kinase domain, a transmembrane domain, and an extracellular ligand-binding domain. The interaction of FGF-2 with the plasma membrane FGFRs induces autophosphorylation of these receptors, then initiates the phosphorylation of tyrosine residues in cytosolic substrates (Fantl et al. 1993).

FGFR-1 is the only high affinity receptor detected so far in the rat heart (Speir et al. 1992; Liu et al. 1995) and plays a vital role in the development of the heart (Wanaka et al. 1991; Mima et al. 1995). Specifically, work by Mima et al. showed that knockout of FGFR-1 function during early avian development results in inhibition of cardiac muscle growth and improper looping of the heart (Mima et al. 1995).

Heparan sulfate proteoglycans (HSPGs) are low-affinity extracellular molecules involved in FGF-2 activity. There is increasing evidence that binding to low-affinity binding sites (HSPGs) on the cell surface plays a important role in the binding of FGF-2 to its high-affinity receptor (Ornitz and Leder 1992; Roghani et al. 1994). Nevertherless, some studies indicate that HSPGs are not absolutely required for FGF-2-induced biological activities, but can enhance these by increasing the binding of FGF-2 to FGFRs (Fannon and Nugent 1996). Some evidence indicates that FGF-2 signaling can be through the HSPG independent pathway, and HSPGs may participate in the internalization of FGF-2 (Quarto and Amalric 1994).

5 Apoptosis

Apoptosis, or programmed cell death (PCD), is an essential mechanism involved in development, and tissue homeostasis, and eliminates the harmful and diseased cells in organisms (Steller 1995; Jacobson et al. 1997). On the other hand, dysregulated apoptosis

has been implicated in many human diseases such as cancer and neurodegenerative diseases (Thompson 1995; Cheng et al. 1998; Cotman and Anderson 1995).

Apoptosis is a genetically regulated cell suicide program characterized by cell shrinkage, plasma membrane blebbing, chromatin condensation, and genomic DNA fragmentation, (Wyllie et al. 1980). Biochemically, apoptotic cells are characterized by reduction in the mitochondrial transmembrane potential, intracellular acidification, production of reactive oxygen species, externalization of phosphatidylserine residues in membrane bilayers, selective proteolysis of a subset of cellular proteins, and degradation of DNA into internucleosomal fragments (Cryns and Yuan 1998).

Apoptotic cells are rapidly eaten by phagocytic cells (macrophages/monocytes, and neighboring cells) before there is any leakage of their contents. Interaction of phosphatidylserine on the apoptotic cell surface with phosphatidylserine receptors on the phagocytosing cells constitutes the major mechanism (Bennett et al. 1995; Fadok et al. 1992).

By contrast, another form of cell death, called cell necrosis, occurs in response to acute injury, toxins, ischemia, and is an uncontrolled process. Cells swell and burst, spilling their contents over their neighbors and eliciting a damaging inflammatory response (Raff 1998).

There are many observable morphological and biochemical differences between apoptosis and necrosis summarized in the following list (adapted from Apoptosis and Cell Proliferation. 2nd edition. BOEHRINGER MANNHEIM 1998)

Morphological features of apoptosis:

- Membrane blebbling, but no loss of integrity.

- Aggregation of chromatin at the nuclear membrane
- Begins with shrinking of cytoplasm and condensation of nucleus.
- Ends with fragmentation of cell into smaller bodies.
- Formation of membrane bound vesicles (apoptotic bodies).
- Mitochondria become leaky due to pore formation involving proteins of the Bcl-2 family.

Morphological features of necrosis:

- Loss of membrane integrity.
- Begins with swelling of cytoplasm and mitochondria.
- Ends with total cell lysis.
- Disintegration (swelling) of organelles.

Biochemical features of apoptosis:

- Tightly regulated process involving activation and enzymatic steps.
- Energy (ATP)-dependent (active process, does not occur at 4°C)
- Non-random mono- and oligonucleosomal length fragmentation of DNA (ladder pattern after agarose gel electrophoresis).
- Prelytic DNA fragmentation.
- Release of various factors (cytochrome C, AIF) into cytoplasm by mitochondria.
- Activation of caspase cascade.
- Alteration in membrane asymmetry (i.e., translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane).

Biochemical features of necrosis:

- Loss of regulation of ion homeostasis.
- No energy requirement (passive process, also occurs at 4°C).
- Random digestion of DNA (smear of DNA after agarose gel electrophoresis).
- Postlytic DNA fragmentation (=late event of death).

5.1 Caspases

Genetic studies of programmed cell death (PCD) in *Caenorhabditis elegans* (*C elegans*) have identified three genes, Ced-3, Ced-4, and Ced-9, that are involved in its cell death. Both Ced-3 and Ced-4 can independently induce cell death, while Ced-9 inhibits cell death (Hengartner et al. 1992; Yuan and Horvitz 1992; Yuan et al. 1993). In mammalian cells, there are counterparts for each of these *C elegans* cell death genes. Bcl-2 is homologous to Ced-9, which inhibits apoptosis (Hengartner and Horvitz 1994). ICE (interleukin-1β-converting enzyme), the first identified mammalian member of the caspase family of cysteine proteases, is homologous to Ced-3 (Yuan et al. 1993). Apaf-1 (apoptotic protease-activating factor-1), which can activate caspase-3, is the homologue to Ced-4 (Zou et al. 1997).

Caspases play an essential role in the induction of internucleosomal DNA fragmentation by activating a latent, cytosolic endonuclease. Caspase-activated deoxyribonuclease (CAD) is normally located in the cytoplasm in an inactive form by binding to an inhibitor (ICAD/DFF-45). This inhibitor suppresses the endonuclease activity of CAD and conceals its nuclear localization signal. During the induction of apoptosis, ICAD/DFF-45 is cleaved by caspase-3-like cysteine protease. This results in

the activation of CAD, leading CAD to the nucleus and subsequent cleavage of the genomic DNA into oligonucleosomal DNA fragmens. this is seen as a typical DNA ladder on agarose gels (Enari et al. 1998; Sakahira et al. 1998).

Another role of caspases in apoptosis is to disassemble cell structure such as the nuclear lamina (Takahashi et al. 1996; Orth et al. 1996), a rigid structure that underlies the nuclear membrane and is involved in chromatin organization. The lamina consists of lamins, which are intermediate filament proteins. During apoptosis, lamins are cleaved at a single site by caspases, causing the lamina to collapse and contributing to chromatin condensation (Rao et al. 1996).

Caspases can cleave the negative regulators of apoptosis, such as the Bcl-2 proteins. It appears that cleavage not only inactivates these proteins, but also produces a fragment that promotes apoptosis (Xue and Horvitz 1997; Adams and Cory 1998).

It is unclear whether caspase proteolysis is responsible for all types of apoptotic cell death, or whether caspase-independent apoptotic mechanisms exist. For example, one study has shown that caspase inhibitors can not prevent apoptosis induced by Bax, a member of the Bcl-2 family (Xiang et al. 1996; McCarthy et al. 1997).

5.2 Bcl-2

The Bcl-2 family of proteins is another central player in apoptosis. At least 15 Bcl-2 family members have been identified in mammalian cells (Adams and Cory 1998). Some can promote cell survival (Bcl-2, Bcl-XL Bcl-W, Mcl-1, and A1), and some can promote cell death (Bax, Bak, Bcl-XS, Bad, Bid, Bik, Bim, Hrk, and Bok).

Bcl-2 has been found on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum (ER), and nuclear envelope. During apoptosis, Bcl-2 proteins regulate the functions of these compartments possibly by modifying the flux of small molecules or proteins (Kroemer 1997; Zamzami et al. 1998; Green and Reed 1998).

Bcl-2 and Bcl-XL can determine the subcellular localization of other apoptosis regulators with which they interact. Bcl-2 targets the protein kinase Raf-1 to mitochondria and Bcl-XL retains the *C elegans* death-trigger protein Ced-4, and presumably its mammalian homologue, Apaf-1, within intracellular membranes, therefore preventing it from entering the nucleus where it will exert its proapoptotic activity (Wu et al. 1997; Chinnaiyan et al. 1997).

The disruption of the mitochondrial transmembrane potential and the release of mitochondrial protease activators are important steps in the apoptotic process. Activation of caspases and Bcl-2 family members enhance mitochondrial permeability transition (PT) by opening the mitochondrial permeability transition pore. Increased mitochondrial permeability transition changes the mitochondrial transmembrane potential, release mitochondrial intermembrane proteins into the cytosol of cells undergoing apoptosis (Kroemer 1997).

Bcl-2 can inhibit the activity of caspase 3, and therefore inhibiting the cleavage of caspase-3 substrate, such as poly (ADP-ribose) polymerase (PARP), subsequent prevents the onset of apoptosis. On the other hand, Bcl-2 family members, such as Bcl-2 and Bcl-XL, two death antagonists, can be cleaved by caspases (Clem et al. 1998; Grandgirard et al. 1998), Bcl-2 by a caspase-3-like protease and Bcl-XL by a caspase-1-like family member. Bcl-2 protein cleavage by caspases generates fragments which have totally

different properties compared to their full-length counterparts; they promote cell death rather than inhibit cell death (Xue and Horvitz 1997; Adams and Cory 1998).

Bcl-2 family members, such as Bcl-2 and Bax, are expressed in cardiomyocytes during ischemia (Misao et al. 1996; Sharov et al. 1996).

The relative amounts or equilibrium between these death antagonists (Bcl-2, Bcl-XL Bcl-W, Mcl-1, A1) and death agonists (Bax, Bak, Bcl-XS, Bad, Bid, Bik, Bim, Hrk, Bok) determines the susceptibility of cells to an apoptotic signal. This ratio is determined by competitive dimerization between selective pairs of antagonists and agonists. Members of the Bcl-2 family interact with each other to form a dynamic equilibrium between homo-hetorodimers, thus determining whether a cell is undergoing apoptosis or not (Kroemer 1997).

5.3 Lamin-B

The lamins are intermediate filament proteins that constitute the lamina, a component of the nuclear envelope that serves to organize the chromatin. Lamins A, B and C are three major components of the lamina (McKeon 1991). Lamin B is expressed early in development and in virtually all somatic cell types (Nigg 1992). Lamin B has an α-helical rod domain to enable assembly into filaments, a nuclear localization sequence to direct filaments to the nucleus, and a carboxy-terminal CAAX box isoprenylation sequence for nuclear membrane targeting (McKeon 1991).

At the onset of mitosis, lamins are disassembled, a process mediated by hyperphosphorylation by $p34^{cdc2}$ kinase at specific sites flanking the α-helical rod domain (Peter et al. 1990). After mitosis, nuclear reassembly requires lamin polymerization,

indicating lamins play an important role in nuclear organization (Heald and McKeon 1990; Nigg 1992).

During apoptosis, lamin disassembly is induced through cleavage by interleukin-1 beta-converting enzyme (ICE) proteases (Earnshaw 1995; Lazebnik et al. 1995). Lamin proteolysis during apoptosis has been reported for several cell types. Etoposide-induced apoptosis in human acute myelogenous leukemia cells (HL-60 cells) is accompanied by extensive lamin B degradation (Kaufmann 1989). Lamin degradation is also observed in apoptosis induced by serum starvation of Ras transformed primary rat embryo cells, where lamin B is degraded into a 46-kDa fragment (Oberhammer et al. 1994). Lamin proteolysis facilitates the activity of the nucleases responsible for the DNA fragmentation that is characteristic of apoptosis. Nuclear breakdown may be required to render the nucleases accessible to proteases or other agents that bring about their activation (Rao et al. 1996).

5.4 Apoptosis in the Heart

Since the cardiac myocyte of the adult heart has lost its ability for a timely and effective regenerative response after injury (Kardami et al 1993), myocyte loss results in replacement of functional muscle by scar tissue, and eventual deterioration of cardiac performance. It is now becoming increasingly clear that myocyte death occurs by both necrosis and apoptosis. Since the apoptotic process is regulated one can envisage the possibility of preventing its development and thus decreasing the extent of cardiac damage. A critical role for the apoptotic process has been proposed for many cardiovascular diseases, including dilated or ischemic cardiomyopathy, myocardial

infarction, myocardial failure, myocarditis, arrhythmias, congenital atrioventricular block (Haunstetter and Izumo 1998).

Extensive research is currently taking place in an attempt to define the triggers and mechanism of cardiomyocyte apoptosis. At this point, cardiac apoptosis seems to follow pathways similar to those described in other cell types: cardiac myocyte apoptosis can be induced by many stimuli, including hypoxia (Tanaka et al. 1994), myocardial infarction (Kajstura et al. 1996), mechanical stretch (Cheng et al. 1995), staurosporine (Yue et al. 1998), tumor necrosis factor α (Krown et al. 1996), aortic constriction (Teiger et al. 1996), increased angiotensin II (Kajstura et al. 1997), atrial natriuretic peptide (ANP) (Wu et al. 1997), and TNF-alpha (Torre-Amione et al. 1995).

The death receptor Fas is unregulated and may mediate apoptosis in cardiomyocytes during ischemia (Yue et al. 1998). Hypoxia is also proposed to induce apoptosis via a p53-dependent mechanism (Long et al. 1997), while Bcl-2 protects adult myocytes from apoptosis (Kirshenbaum and de Moissac 1997). Some evidence for potential therapeutic interventions targeting the mechanism of apoptosis was provided by Yaoita et al (1998) who demonstrated that caspase inhibitors led to a reduction in infarct size after myocardial infarction.

5.5 Chromatin Condensation in Apoptosis

Apoptotic chromosome condensation is not driven by the histone phosphorylation mechanism that is used for mitotic chromosome condensation. During the early stages of apoptosis, euchromatin is hypersensitive to nuclease degradation due to the pre-existing levels of acetylated histone H3 and H4. Normally, these acetylated histones are located at

the transcription competent region of chromatin. Degradation of the nuclease-sensitive acetylated chromatin at the early stage of apoptosis is accompanied by the degradation of lamins and reorganization of an intranuclear protein matrix. These events cause the collapse of the nucleus. Aggregation of heterochromatin produces the appearance of apoptotic chromatin condensation. Later, this aggregated heterochromatin is cleaved by nucleases, and causes the oligonucleosomal DNA fragmentation, which is believed to be the hallmark of apoptosis (Hendzel et al. 1998).

6 Mitotic Chromosome Condensation

Mitotic chromosome condensation is one of the cell division processes. In this process, the relaxed chromatin of interphase is condensed to allow the correct segregation of sister chromatids into two genetically identical cells. Mitotic chromosome condensation is believed to have two functions (Koshland and Strunnikov 1996). One is to help resolve chromatin tangles between sister chromatids; these tangles occur during DNA replication (Weaver et al. 1985). The second function is to package the interphase relaxed chromosomes into compacted mitotic chromosomes. The interphase chromosomes are longer than the length of the dividing cell, so packaging interphase chromosomes would help to avoid the cleavage of chromosome during cytokinesis, therefore maintaining the integrity of genetic information during mitosis (Koshland and Strunnikov 1996; Hirano 1995). However, the molecular mechanism of mitotic chromosome condensation is poorly understood (Wei et al. 1999)

Three classes of proteins are believed to be involved in mitotic chromosome condensation (Koshland and Strunnikov 1996). One class is histone proteins, the second

class is topoisomerase II, and the third class is SMC (structural maintenance of chromosomes) proteins.

Histone proteins are associated with DNA to form chromatin. The four core histones (H2A, H2B, H3 and H4) and DNA form nucleosomes, the basic unit of chromatin, and compact the DNA (Luger et al. 1997). Linker histone H1 binds to the nucleosomes and further compacts the chromatin (Vignali and Workman 1998). Early experiments indicated that the phosphorylation of histone H1 plays an important role in mitotic chromosome condensation (Bradbury 1992). However, other experiments have demonstrated that the phosphorylation of histone H1 is not required for mitotic chromosome condensation (Guo et al. 1995; Ohsumi et al. 1993), thus the function of histone H1 in mitotic chromosome condensation remains to be determined. More recent studies now suggest that histone H3 phosphorylation plays a important role in mitotic chromosome condensation (Van Hooser et al. 1998; Hendzel et al. 1997; Wei et al. 1999).

Topoisomerase II is an enzyme that can catalyze the creation of a transient double-strand breaks in the DNA, which allows the passage of one DNA strand through another (Hsieh and Brutlag 1980), Topoisomerase II is therefore a key player for the structural changes during activation for the proper condensation of mitotic chromosome (Earnshaw et al. 1985).

Structural maintenance of chromosomes (SMC) proteins are the newest proteins found to be involved in mitotic chromosome condensation. Mutation of SMC proteins caused defects in mitotic chromosome condensation in yeast (Saka et al. 1994;

Strunnikov et al. 1995). Immunodepletion of SMC proteins inhibited chromosome condensation in *Xenopus* egg extracts (Hirano and Mitchison 1994).

Significance of Mitosis for cardiac myocytes: As mentioned earlier, after birth cardiac myocytes appear to become inhibited in their ability for proliferation and thus for regeneration. The ability for cell division however may not have been lost irreversibly, as indicated by work from Anversa's laboratory (Kajstura et al. 1996). Mitotic condensation of chromosomes is an essential feature of cell division, and therefore understanding its regulation in the context of the cardiomyocyte may lead to a means of facilitating progression through the cell cycle, and thus regeneration. At this point, however, there is no information about control of chromosome condensation in cardiac myocytes.

7 Hypothesis

Previous studies in our laboratory had indicated that overexpression of rat HMW FGF-2, but not LMW FGF-2, in rat and chicken myocytes caused a distinct nuclear phenotype of condensed chromatin, separated in several 'clumps' (Pasumarthi et al. 1994; Pasumarthi et al. 1996), via an intracrine pathway. Distinct effects on chromatin are of great interest in that they may be part of any of several cellular mechanisms, including apoptosis, mitosis and gene expression. Although a number of other laboratories have presented evidence for differential effects of the HMW versus the LMW FGF-2 on cells (Vagner et al. 1996; Piotrowicz et al. 1997; Couderc et al. 1991; Quarto et al. 1991; Kamiguchi et al. 1996; Galy et al. 1999), there has been no previous report on specific effects on chromatin. There were several important differences however between the

system used in our previous studies (Pasumarthi et al. 1994; Pasumarthi et al. 1996) and those used by other investigators:

(1): Data by Pasumarthi et al (1994; 1996) were obtained using rat FGF-2, while all other studies by other investigators used human FGF-2.

(2): Previous studies have used transformed cell lines (such as SK-Hep-1, HeLa, COS-7, MCF-7 cells (Vagner et al. 1996; Florkiewicz et al. 1991), or NIH 3T3 cells (Bikfalvi et al. 1995), in contrast to the primary cultures of cardiac myocytes used by Pasumarthi et al (1994; 1996).

(3), Previous studies have used cell lines stably overexpressing the FGFs, therefore other investigators have selected for surviving, fast proliferating cells; Pasumarthi et al (1994; 1996) have used transient gene transfer.

In this work, we have thus first addressed the hypothesis that human FGF-2, sharing 82% homology with rat FGF-2 at the CUG-extension, will elicit the same effects as its rat counterparts on the nuclei of cardiac myocytes. This would indicate whether the previous observations were species-specific or reflected general properties of FGF-2.

In addition, and since chromatin condensation and nuclear fragmentation are features of apoptosis or mitosis, we have examined the hypothesis that the HMW FGF-2 induced nuclear phenotype is following an apoptosis-like, or a mitosis-like mechanism.

To address the above hypothesis, we have used primary cultures of rat cardiomyocytes and transient gene transfer with cDNAs expressing humna HMW or LMW FGF-2. Due to the largely qualitative nature of our studies, we have made

extensive use of immunofluorescence and localization of apoptosis and/or mitosis 'markers'.

MATERIALS AND METHODS

1 Plasmids

The human FGF-2 cDNAs were generous gifts of Dr. RZ Florkiewicz (CIBLEX Corporation, San Diego, California). Human FGF-2 cDNAs were cloned downstream of cytomegalovirus promoter in the pcDNA 3.0 expression vector. The cDNAs coding for three HMW FGF-2 isoforms (pcDNA3-363) and all FGF-2 isoforms (pcDNA3-14) were subcloned into the Eco R1 site, respectively; the cDNA codes the 18 kDa isoform (pcDNA3-18) was subcloned into the Xho I site. In those three human FGF-2 expression vectors, pcDNA3-14 encodes all FGF-2 isoforms, pcDNA3-363 encodes the three HMW FGF-2 isoforms, and pcDNA3-18 encodes the 18 kDa isoform. pcDNA3-363 and pcDNA3-18 were used in our experiments. Bcl-2 cDNA is a generous gift of Dr. Lorrie Kirshenbaum (University of Manitoba).

2 Cell Culture

Neonatal rat cardiac myocyte cultures were prepared as described previously (Doble et al. 1996). Briefly, the hearts were isolated from 1-day-old Sprague-Dawley rats and were minced in Ham's F-10 medium; the cells were dissociated in a water-jacked spinner flask (37°C) using a combination of trypsin (GIBCO BRL. Burlington, ON, Canada) and DNase I (Sigma-Aldrich, Oakville, ON, Canada). Cardiac myocytes were purified on a discontinuous Percoll gradient. The cardiac myocytes were suspended in Ham's F-10 medium containing 10% fetal bovine serum (FBS), 10% horse serum (HS), and antibiotic (1,000 U/ml penicillin, 1 mg/ml streptomycin). Cardiomyocytes were

counted with a hemocytometer and plated on collagen-coated plates at a density of $0.5x10^6$ cells per 35-mm culture dish. All cultureware was purchased from Corning (Fisher Scientific, Nepean, ON, Canada). Culture medium was from GIBCO BRL.

3 Transient Gene Transfer

Cardiac myocytes were transfected using the calcium phosphate-DNA precipitation method as previously described (Pasumarthi et al. 1996) with modification (Xu et al. 1992). Briefly, neonatal rat cardiac myocytes were plated on collagen-coated plates at a density of 0.5×10^6 per 35 mm culture dish. The cells were transfected 24 h after plating. The cell density was at 50-60% confluence. Two to three hours prior to the transfection, Ham's F-10 medium was removed, the cells were rinsed twice with warm (37° C) Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF-PBS), and switched to DMEM medium containing 10% FBS, antibiotic (1,000 U/ml penicillin, 1 mg/ml streptomycin). For each 35 mm dish transfection, 5 μg of plasmid DNA was used to make up 175 μl calcium/DNA mixture with 20μl of 2M $CaCl_2$ and nuclease-free water in a sterilized 1.5 ml microcentrifuge tube. 175 μl of 2×HEPES-buffered saline (280mM NaCl, 50 mM HEPES PH7.1, and 1.5 mM Na_2HPO_4) was placed in a separate sterile 1.5 ml microcentrifuge tube, and 175 μl of DNA/Calcium solution were added drop-wise to the 2×HBS while bubbling the 2×HBS with a Pasteur pipette. The calcium phosphate-DNA precipitates were allowed to form at room temperature for 1 min, and then 350 μl of mixed solution was added drop-wise to a 35-mm culture dish. Cells were incubated with the precipitates for 24 h in a humidified incubator (37°C, 5% CO_2), washed three times with CMF-PBS to remove remaining precipitates, and maintained in DMEM

medium containing 10% FBS, antibiotic (1,000 U/ml penicillin, 1 mg/ml streptomycin) for a further 48 h in a humidified incubator (37° C, 5% CO_2) before processing.

The co-transfections of human HMW FGF-2 and Bcl-2, human HMW FGF-2 and LMW FGF-2 were done following the same procedure. 5 μg of each plasmid DNA was used for the co-transfection.

4 Analysis of DNA Fragmentation

Genomic DNA was isolated and detected as described previously (Yue et al. 1998). Myocytes from four 35 mm dishes were washed 2 times with cold PBS, then scraped off the culture dishes and move to a 1.5 ml microfuge tube, and centrifuged at 360g for 5 min at 4° C. The cell pellet was then resuspended in 200μl of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, and 0.2 mg/ml proteinase K) and incubated at 55°C for 3 hours. After that, the lysate was further incubated with RNase A (10 μg/ml) for 1 hour at 37°C. DNA was extracted by phenol/chloroform/isoamyl alcohol extraction. DNA was loaded onto a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. DNA fragments were visualized under UV light.

Cultured rat neonatal cardiac myocytes were treated with 0.5 μM or 1.0 μM of staurosporine for 24 hour to induce apoptosis, as a positive control (Yue et al. 1998). Staurosporine-induced cardiac myocyte apoptosis involves activation of caspases, mainly caspase-3 (Yue et al. 1998).

5 *In-Situ-End* Labeling for Detection of Apoptotic Myocytes

In situ detection of apoptotic cells was performed by using an *In Situ* Cell Death Detection Kit (Fluorescein). (Boehringer Mannheim Biochemicals) following the manufacturer's instruction. Briefly, cardiac myocyte cultures were rinsed three times with PBS 48 h after transfection, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, then rinsed again with PBS, permeabilized on ice with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min, rinsed thoroughly with PBS, and then incubated with the TUNEL reaction mixture for 60 min at 37° C. To detect the overexpression of FGF-2, coverslips were incubated first with guinea pig anti-FGF-2 antibodies (20 μg/ml) in 1% BSA in PBS overnight at 4° C. The FGF-2 was visualized with guinea pig Texas red antibodies (1:50, Jackson Immunoreasearch Laboratories, Inc. USA) diluted in 1% BSA in PBS. Cellular DNA was stained with 0.0125% Hoechst dye 33342 for 1 min (Kardami and Fandrich 1989). Coverslips were then mounted on slides using ProLong antifade mounting medium (Molecular Probes, Eugene, Oregon), cells were viewed and photographed with a Nikon Diaphot epifluorescence microscope equipped with appropriate filters.

6 Immunofluorescence

Rabbit anti-FGF-2 antibodies (Kardami et al. 1991) and guinea pig anti-FGF-2 antibodies (characterized in the Kardami laboratory, unpublished) used in present study can recognize all forms of FGF-2. Monoclonal antibody against striated muscle myosin (MF-20 hybridoma) was obtained from the Development Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins

University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract No 1-HD-6-2915 from the NICHD. Polyconal rabbit antibodies to lamin-B were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat polyconal antibodies to Bcl-2 were purchased from Calbiochem-Novabiochem (San Diego, CA)

Cardiac myocytes grown on collagen-coated coverslips were fixed and permeabilized as described previously (Doble et al. 1996) 48 h after transfection, the cells were fixed with 1% paraformaldehyde in PBS for 15 min at 4°C, permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 15 min at 4°C, and rinsed thoroughly with PBS. The cells were then processed for immunostaining.

Double immunofluorescence labeling for FGF-2 and myosin was done as follows: coverslips were incubated with polyconal rabbit FGF-2 antibody at 1:2000 dilution and monoclonal MF-20 antibody at 1:100 dilution in 1% (W/V) bovine serum albumin in PBS overnight at 4°C. This was followed by rinsing , and then incubation with biotinylated anti-rabbit immunoglobulins (1:20, Amersham, Arlington Heights, IL) for 1 h at room temperature. After further rinsing, coverslips were incubated with fluorescein-conjugated streptavidin (1:20, Amersham, Arlington Heights, IL) and Texas red conjugated anti-mouse IgG (1:20, Amersham, Arlington Heights, IL) for 1 h at room temperature. Cellular DNA was stained with 0.0125% Hoechst dye 33342 (Calbiochem-Novabiochem, San Diego, CA) in PBS for 1 min as described before (Kardami and Fandrich 1989). Coverslips were then mounted on slides using ProLong antifade mounting medium (Molecular Probes, Eugene, Oregon) and viewed and photographed with a Nikon Diaphot epifluorescence microscope equipped with appropriate filters.

Double immunofluorescence labeling for Lamin B and FGF-2 in human HMW FGF-2 transfected cardiac myocytes was done as follows: coverslips were incubated with goat polyclonal Lamin B antibody (Santa Cruz, CA) at a 1:100 dilution and rabbit polyclonal FGF-2 antiserum at 1:2000 dilution in 1% BSA in PBS overnight at 4°C, then with biotinylated anti-rabbit immunoglobulins (1:20, Amersham,) for 1 h at room temperature, followed by incubation with fluorescein conjugated to streptavidin (1:20, Amersham) and Texas red conjugated anti-goat IgG (1:50, Jackson Immunoreasearch Laboratories, Inc. USA) for 1 h at room temperature. Nuclear staining, coverslips mounting and visualization were carried out as described above.

Double immunofluorescence labeling for Bcl-2 and FGF-2 in human HMW FGF-2 and Bcl-2 co-transfected cardiac myocytes was done as follows: coverslips were incubated with polyclonal rabbit anti-Bcl-2 antibody (Calbiochem-Novabiochem, San Diego, CA) at 1:100 dilution and guinea pig anti-FGF-2 IgG (20 μg/ml) in 1% (W/V) BSA in PBS overnight at 4°C. Samples were then incubated with biotinylated anti-rabbit immunoglobulins (1:20, Amersham) for 1 h at room temperature, followed by incubation with fluorescein conjugated streptavidin (1:20, Amersham) and Texas red conjugated anti-guinea pig IgG (1:50, Jackson Immunoreasearch Laboratories, Inc. USA) for 1 h at room temperature. Nuclear staining, coverslips mounting and visualization were carried out as described above.

7 Phosphorylated Histone H1 and Histone H3 Staining

Indirect immunofluorescence for histone H1 and H3 were carried out as described previously (Lu et al. 1994). Rabbit antisera to phosphorylated histone H1 or H3 were

generously supplied by Dr. J R. Davie (University of Manitoba, Canada). Cardiac myocytes were fixed with 2% formaldehyde in PBS for 15 min at room temperature 48 h after transfection, permeabilized with anhydrous methanol for 15 min at room temperature, rinsed twice with 10% FBS in PBS, following by further washes with PBS two times. Simultaneous labeling for FGF-2 and phosphorylated histone H1 or histone H3 was carried out by incubation with guinea pig anti-FGF-2 IgG (20μg/ml, characterized in the Kardami laboratory, unpublished) and anti-phosphorylated histone H1 serum (1:300 dilution) or anti-phosphorylated histone H3 serum (1:300 dilution) in 1% BSA in PBS overnight at 4°C. Then coverslips were incubated with biotinylated anti-rabbit immunoglobulins (Ig, 1:20, Amersham, Arlington Heights, IL) for 1 h at room temperature, followed by incubation with Texas red conjugated anti-guinea pig IgG (1:50, Jackson Immunoreasearch Laboratories, Inc. USA) and fluorescein conjugated to streptavidin (1:20, Amersham) for 1 h at room temperature. Cellular DNA was stained with 0.0125% Hoechst dye 33342 (Calbiochem-Behring, San Diego, CA) in PBS for 1 min as described before (Kardami and Fandrich 1989). Coverslips were mounted on slides using ProLong antifade mounting medium (Mollecular Probes, Eugene, Oregon) and viewed and photographed with a Nikon Diaphot epifluorescence microscope equipped with appropriate filters

8 Statistical Analysis

A paired Student's t-test was used for the statistical analysis of the data. Square coverslips (22×22 mm) were divided into 16 equal triangles. One field was observed per triangle: a total of 800 cells were scored per coverslip, and three coverslips were included

per experiment (n=3). Each experiment was repeated 3 times. In the comparison of average percentage of DNA clumping between two values, a difference was considered statistically significant when the P value was <0.05.

RESULTS

1 Effect of HMW FGF-2 on Nuclear Morphology

A diagram of the different plasmids coding for human FGF-2 used for our studies is shown in Fig 1. The cDNAs coding for human HMW and LMW FGF-2, driven by the CMV promoter, were introduced into rat cardiac myocytes by transient gene transfer, using a modified calcium phosphate method that resulted in 10-20% transfection efficiency. Transfection efficiency were determined by CMVp.ß-gal transfection of neonatal rat cardiac myocytes. According to the ß-gal staining, 10-20% (15±4, n=6) of cells in cultures were transfected with CMVp.ß-gal compared with cultures transfected with the vector plasmid, where no staining was observed. Similar levels of transfection efficiency were obtained by counting the percentage of overexpressing FGF-2 cardiac myocytes in cultures with immunofluorescence microscope. Expression of products of the introduced genes within individual cells was evaluated by immunofluorescence staining for FGF-2, using two different preparations of anti-FGF-2 antibodies, one prepared in rabbit (serum S2, characterized fully by Kardami et al. 1991), and the other in guinea pig (characterized in the Kardami laboratory, unpublished). Both antibody preparations recognize all forms of FGF-2. They were used at high enough dilutions to achieve negligible staining of endogenous FGF-2 while clearly detecting overexpressing cells.

As indicated by intense fluorescence staining, HMW FGF-2 was localized to the nuclei of all overexpressing cells (Figs 2, 3, 8, 9, 10). In addition a proportion (20%) of cells showed staining near the nucleus, possibly the endoplasmic reticulum (Fig.2). LMW

FGF-2 was also localized to the nucleus, but also to the cytoplasm of overexpressing cells (Fig.2).

All cultures were counterstained for DNA to localize nuclei. As was seen before with the rat HMW FGF-2 (Pasumarthi et al. 1994; Pasumarthi et al. 1996), a substantial proportion of myocytes overexpressing human HMW FGF-2 displayed a distinct nuclear phenotype: the chromatin appeared condensed (as indicated by the brighter Hoechst staining intensity) in separate irregular fragments ("clumps") of different sizes. A typical image is shown in Fig 2B. This nuclear morphology was very rarely (<2.5%) seen in myocytes overexpressing LMW FGF-2, and was not discernible in myocytes transfected with vector.

A very small proportion (<1%) of HMW FGF-2 overexpressing myocytes displayed multinucleation (Fig.3). This has also been observed previously for rat HMW FGF-2 (Pasumarthi et al. 1996). This phenotype was never observed in cultures transfected with LMW FGF-2 or with vector.

2 Effect of Apoptosis Inhibitors (Bcl-2, caspase inhibitors, and LMW FGF-2) on the HMW FGF-2 -Induced Nuclear Phenotype.

Myocytes were simultaneously transfected with the cDNAs for Bcl-2, an anti-apoptotic protein, and HMW FGF-2. Expression of the corresponding proteins was assessed by double immunofluorescence studies. Anti-Bcl-2 antibodies produced negligible staining in myocyte cultures transfected with vector alone (data not shown);

presumably levels of endogenous Bcl-2 are below the sensitivity limit of the antibodies used. Anti-Bcl-2-positive myocytes were however detected in cultures transfected with the cDNA coding for Bcl-2. As shown in Fig.4, anti-Bcl-2 staining was detectable in the perinuclear region of the cells. We examined the nuclear morphology of myocytes overexpressing both Bcl-2 (green) and HMW FGF-2 (red). As shown in Fig.4, C and F, overexpression of Bcl-2 did not prevent chromatin clumping induced by HMW FGF-2. A quantitative assessment of these data is shown in Fig.5: the percentage of 'clumped' nuclei remained unaffected by Bcl-2. Myocytes overexpressing Bcl-2 but not HMW FGF-2 did not display the 'clumped' phenotype (data not shown).

Myocytes were treated with the broad spectrum caspase inhibitor Z-VAD-fmk (100 μM), or the caspase-3-specific inhibitor Z-DEVD-fmk (100 μM), for 30 min prior to gene transfer of the HMW FGF-2 cDNA, throughout the gene transfer period, and for 48 h after gene transfer. The percentage of 'clumped' nuclei in inhibitor-treated myocytes (overexpressing HMW FGF-2) remained identical to that from the untreated myocytes (overexpressing HMW FGF-2, Fig.5).

Myocytes were transfected simultaneously with HMW and LMW FGF-2. The percentage of 'clumped' nuclei remained unchanged between cultures overexpressing HMW FGF-2 alone or both HMW and LMW FGF-2. Myocytes overexpressing LMW FGF-2 alone had very low incidence of nuclear clumping, (Fig.5).

3 Effect of HMW FGF-2 on DNA Degradation, TUNEL Staining and Lamin B Localization

Genomic DNA was isolated from myocytes transfected with vector, HMW FGF-

2, and myocytes treated with staurosporine to induce apoptosis, and analyzed by agarose gel electrophoresis. Results are shown in Fig.6. Staurosporine induced the classic pattern of apoptotic ladder. It was not possible to detect a laddering pattern in the DNA from HMW FGF-2-treated myocytes.

Triple fluorescence labeling for FGF-2, TUNEL, and nuclear DNA was used to investigate whether the HMW FGF-2-induced nuclear clumps were positive for TUNEL. The majority (>80%) of myocytes overexpressing HMW FGF-2 and displaying the 'clumped' phenotype were negative staining for TUNEL (Fig.7). A low percentage (<1%) of overexpressing myocytes did stain positive for TUNEL (Fig.7), this percentage was similar to the baseline level of TUNEL-positive nuclei present in control or vector-transfected cultures.

Cardiac myocytes overexpressing HMW FGF-2 were simultaneously stained for FGF-2 and lamin B, a nuclear cytoskeleton component that contributes to the integrity of the nuclear envelope (McKeon 1991; Nigg 1992). Loss of lamin staining around the nucleus indicates disruption of the nuclear membrane, a phenomenon seen in apoptosis as well as mitosis. Typical findings are shown in Fig.8. Perinuclear anti-laminB staining was clearly retained in myocytes overexpressing HMW FGF-2 and displaying the 'clumped' chromatin phenotype.

4 Effect of HMW FGF-2 overexpression on Histone H1, H3 Phosphorylation.

Phosphorylation of histones H1 and H3 has been shown to correlate strongly with the mitotic nuclear phenotype condensation (Bradbury 1992; Chadee et al. 1995; Van

Hooser et al. 1998; Hendzel et al. 1997; Wei et al. 1999). Double immunofluorescence labeling of cardiac myocytes overexpressing HMW FGF-2 with antibodies specific for the phosphorylated forms of H1 and H3 (rabbit polyclonal, anti-P-H1, anti-P-H3), and for FGF-2 (guinea pig polyclonal), was used to examine the state of histone phosphorylation in myocytes overexpressing HMW FGF-2 and displaying the 'clumped' nuclear phenotype.

Typical results obtained for the phosphorylation of histone H1 are shown in Fig.9: At the concentration used, the anti-P-H1 antibody produced a generalized cytosolic staining, strong staining of mitotic chromosomes (Fig.9A) and strong staining of some nuclei (Fig.9D). None of the nuclei in cells overexpressing HMW FGF-2 stained strongly with the anti-P-H1, in any of the fields observed (10 different fields per coverslip, three coverslips). The intensity of the anti-P-H1 nuclear staining appeared similar between myocytes overexpressing HMW FGF-2 and non-overexpressing, non-mitotic myocytes.

Similar results were obtained using the anti-P-H3 antibodies. These antibodies also produced intense staining of obviously mitotic chromosomes and of some nuclei (Fig.10A). None of the nuclei in cells overexpressing HMW FGF-2 stained strongly with the anti-P-H3, in any of the fields observed (10 different fields per coverslip, three coverslips). The intensity of the anti-P-H3 nuclear staining was similar between myocytes overexpressing HMW FGF-2 and non-overexpressing, non-mitotic myocytes.

DISCUSSION

1 Human FGF-2 versus rat FGF-2

Although the 'short', AUG-initiated, 18 kDa FGF-2 is highly (98%) homologous between the human and rat species (Shimasaki et al. 1988), and there are no differences in the receptor-mediated biological activity between the two species, there are some differences between the N-terminal extension resulting from CUG-translation. The N-terminal extensions of human FGF-2 are comprised of 41, 46 and 55 amino acids, compared to 26 and 34 amino acids of the rat HMW FGF-2 (Shimasaki et al. 1988; Brigstock et al. 1990). When the N-terminal sequences are aligned for maximal homology, 82% of the rat amino acid sequence is identical to the human sequence (Brigstock et al. 1990); furthermore, the human HMW FGF-2 has 2 copies of the sequence GGRG (linked to nuclear targeting and retention) while the rat has one. Before embarking on studies aimed at understanding the mechanism by which HMW FGF-2 induced the condensed chromatin phenotype, it was important to address the first of the points listed above, i.e. to examine whether human HMW FGF-2 had similar effects as its rat counterpart on cardiac myocytes.

We obtained the human FGF-2 cDNA, driven by the CMV promoter and modified it so that it would code exclusively for the CUG-initiated (HMW FGF-2) or AUG-initiated (LMW FGF-2) species of FGF-2 (Florkiewicz and Sommer 1989). These constructs have been shown to produce 23-25 kDa and 18 kDa FGF-2, respectively (Fig 1). Effective expression in our system was ascertained qualitatively, by immunolocalization (Fig 2). As we have demonstrated previously (Pasumarthi et al. 1994; Pasumarthi et al. 1996), overexpressing myocytes stain very brightly for FGF-2

and can be clearly differentiated from non-overexpressing cells; bright staining of 5-10% of myocytes represents significant increases in total HMW or LMW FGF-2 protein quantitated by western blotting (Pasumarthi et al. 1994; Pasumarthi et al. 1996). Overexpression of the human HMW (but not LMW) FGF-2 in cardiomyocytes produced chromatin clumping and fragmentation in a manner morphologically indistinguishable to that caused by the rat HMW FGF-2. Subcellular localization of the HMW and LMW FGF-2 also indicated a qualitatively similar pattern of localization as for the rat isoforms: The HMW FGF-2 was found predominantly in the nucleus, while LMW FGF-2 was localized in the nucleus and the cytosol of cardiomyocytes. As seen previously for rat FGF-2, usually 20-30% of HMW FGF-2 overexpressing myocytes presented the 'clumped' phenotype (Fig 2), although in some experiments the percentage was as high as 50%. One can speculate that the extent of manifestation of the phenotype is a function of the level of expression that may vary between experiments, but also of the growth and metabolic state of the individual cell. It is also possible that there exist heterogeneous populations of cardiac myocytes in our primary cultures. Further experiments are needed to address these issues.

A small number of myocytes overexpressing human HMW FGF-2 displayed multinucleation. Multinucleated cardiac myocytes are reported to represent 5% of ventricular myocytes in the adult rat heart (Kellerman et al. 1992) and there are similar examples in the human heart (Shozawa et al. 1990). HMW FGF-2 overexpressing myocytes were observed containing 3-6 apparently full size nuclei; all or none of these nuclei had the 'clumped' phenotype. Multinucleation can result from caryokinesis without cytokinesis, followed perhaps by another cycle of caryokinesis by some of the nuclei.

Amitotic division may also create the phenotype of multinucleation. At this point we have no information about the cause of multinucleation in our system; our observations can be taken as additional evidence that the human FGF-2 has the same properties as its rat counterpart (Pasumarthi et al. 1996). One can speculate that the distinct properties of HMW FGF-2 in the nucleus likely contribute towards multinucleation.

2 HMW FGF-2 and Apoptosis

Having established that human HMW FGF-2 caused a distinct nuclear phenotype in cardiomyocytes, we proceeded to investigate the potential mechanism for the observed effect. We thus investigated whether HMW FGF-2 triggered an apoptosis-like process, since the nuclear phenotype it produced at the level of epi-fluorescence microscopy was morphologically similar to that observed in nuclei undergoing apoptosis. Specifically, chromatin stained brighter with the fluorescent dye Hoechst 33342, indicating condensation, and appeared in distinct segments, or 'clumps', inside the nucleus.

A widely accepted indicator of apoptosis is the presence of a ladder-like pattern of DNA degradation in agarose gels (Wyllie et al. 1980; Arends et al. 1990). However this method has the disadvantage of relatively low sensitivity: DNA degradation is detectable by this method only when a high proportion of apoptotic cells is present (Rotello et al. 1989; Desmouliere et al. 1995). Analysis of DNA from HMW FGF-2 overexpressing cultures failed to detect a DNA ladder, while clearly detecting the ladder pattern in the DNA from staurosporine-treated myocytes, used as positive control. It would appear therefore that the chromatin segmentation that observed in HMW FGF-2 overexpressing myocytes did not reflect apoptotic DNA cleavage. However, as mentioned above, the

sensitivity of detection may have been too low. Cardiac myocytes in primary cultures are difficult to transfect, and most published studies that have used non adenovirally-mediated gene transfer have reported 1-10% transfection efficiency. Our laboratory has improved this to 10- 20% by following a modified calcium-phosphate-mediated procedure (Xu et al. 1992), and this has facilitated the present work. Nevertheless, it may still have not been sufficient to detect the DNA ladder characteristic of apoptosis. Very rare exceptions have been described where morphological features of apoptosis are not accompanied by oligonucleosomal DNA cleavage (Cohen et al. 1992). It is therefore theoretically possible that the HMW FGF-2 phenotype may be apoptosis without oligonucleosomal DNA cleavage.

Since studies based on biochemical quantitative analysis may not be sensitive enough, we have used a variety of qualitative, morphological criteria to detect the existence of an apoptosis-like process. These included TUNEL staining, detection of perinuclear lamin B, and use of anti-apoptotic manipulations.

Individual apoptotic cells can be detected by *in situ* enzymatic labeling of apoptosis-induced DNA strand breaks. DNA polymerase and terminal deoxynucleotidyl transferase (TdT) are used for the incorporation of labeled nucleotides (dUTP) to DNA strand breaks in situ (Gavrieli et al. 1992). TUNEL (TdT-mediated dUTP nick end labeling) detection was therefore used in our system. Although cardiomyocyte nuclei rendered apoptotic by staurosporine stained positive for TUNEL (unpublished observations), 'clumped' nuclei in HMW FGF-2 overexpressing cells did not stain for TUNEL, indicating the absence of strand breaks and therefore of an apoptosis-like DNA fragmentation.

The lamins are intermediate filament proteins present in the nucleus, where they are the main structural component of the nuclear lamina. The nuclear lamina serves to organize the chromatin (McKeon 1991). Lamin B is one member of the lamin family expressed in virtually all cells (Nigg 1992). During apoptosis, lamins are cleaved by ICE proteases, a phenomenon observed in several cell types: anti-lamin immunostaining disappears from the periphery of the apoptotic nuclei (Kaufmann 1989; Oberhammer et al. 1994; Earnshaw 1995). It is believed that a function of lamin proteolysis is to facilitate activation of nucleases responsible for DNA fragmentation in apoptosis. We thus used simultaneous immunostaining for lamin B and FGF-2, as well as Hoechst nuclear stain, to examine whether the nuclear lamina was disrupted in HMW FGF-2 overexpressing myocytes that had the 'clumped' nuclear phenotype (Fig.8). All myocytes presenting the 'clumped' phenotype had an apparently intact anti-lamin B perinuclear staining, a finding consistent with a non-apoptotic mechanism of nuclear condensation.

A number of manipulations have been shown to prevent development of the apoptosis in many different systems. ICE proteases are widely considered as central to the apoptotic process (Enari et al. 1998; Nicholson et al. 1995; Tewari et al. 1995; Kuida et al. 1995). Inhibition of caspase-3 has been shown to prevent apoptosis in cardiomyocytes (Yue et al. 1998; Malhotra and Brosius 1999). We used a 'general' ICE-inhibitor Z-VAD-fmk, and a specific caspase-3 inhibitor Z-DEVD-fmk, at concentrations shown to be effective on cardiomyocytes by other investigators (Malhotra and Brosius 1999). These treatments were unable to prevent the appearance of the 'clumped' nuclear phenotype in HMW FGF-2 overexpressing cells, suggesting that the phenotype did not require caspase activation, and thus it did not represent caspase-dependent apoptosis.

Pilot studies (unpublished observations) were also done to examine whether HMW FGF-2 overexpression was accompanied by activation of caspase-3, assessed by the appearance of the 'active' 17 kDa fragment. We were unable to detect the 17 kDa caspase fragment by western blotting with specific antibodies, a finding indicative of absence of caspase-3 activation, and of caspase-3-dependent apoptosis.

Bcl-2, a 26-kDa protein distributed to mitochondrial membranes, endoplasmic reticulum and the nuclear envelope (Reed 1994) is also considered to prevent apoptosis by many stimuli (Tsujimoto 1989; Alnemri et al. 1992; Kluck et al. 1997). We thus used overexpression of Bcl-2, achieved by transient gene transfer, to demonstrate that the segmented 'clumped' nuclear phenotype induced by HMW FGF-2 was not prevented by the simultaneous overexpression of Bcl-2. It should be noted that in cultures transfected with both Bcl-2 and HMW FGF-2, most of the cells overexpressing HMW FGF-2 were also found to overexpress Bcl-2 (by dual immunolabeling), although cells over-expressing either FGF-2 or Bcl-2 (but not both) were also seen. Overall, the inability of Bcl-2 expression to prevent the 'clumped' phenotype is consistent with the notion that the mechanism is not related to Bcl-2 preventable apoptosis. Alternatively, Bcl-2 levels may have been insufficient for apoptosis prevention. Future experiments will employ adenovirally-driven Bcl-2 gene transfer to address this issue.

Finally, as reviewed in the introduction, LMW FGF-2, acting via plasma membrane receptors, is reported to protect many cells, including cardiac myocytes. We therefore overexpressed LMW FGF-2 as well as HMW FGF-2, to examine whether there was prevention of the 'clumped' phenotype. We have previously demonstrated that overexpression of LMW FGF-2 in cardiac myocytes by transient gene transfer results in

receptor-mediated effects on the cells caused by the release of the growth factor into the medium (Pasumarthi et al. 1996). Overexpression of LMW FGF-2 (or addition of exogenous FGF-2 at 50 ng/ml, unpublished data) did not affect the HMW FGF-2 induced phenotype, indicating absence of LMW FGF-2-preventable apoptosis.

Taken individually, the above criteria do not constitute absolute 'markers' of apoptosis. It is possible to have non-Bcl-2-dependent, even non-caspase-dependent apoptosis, and LMW FGF-2 may be effective in preventing apoptosis under certain conditions only; one can even hypothesize that our method may not have been sensitive enough to detect changes in lamin B in association with the nuclear membrane. Lack of TUNEL staining, however, clearly indicates absence of DNA strand breaks, and thus argues more convincingly that the condensed and segmented chromatin phenotype in HMW FGF-2 expressing myocytes does not result from any known apoptotic mechanism.

3 HMW FGF-2 and Mitosis

FGF-2 is a potent mitogen. Overexpression of growth factors in normal cells tends to result in loss of growth control and the appearance of various degrees of transformation. NIH 3T3 cells overexpressing HMW FGF-2 exhibit a transformed phenotype in vitro (Arese et al. 1999). We have shown that HMW FGF-2 stimulated cardiomyocyte proliferation via an autocrine/paracrine, receptor-mediated mechanism, and that its effects on the nuclear phenotype were independent of the proliferative effect (Pasumarthi et al. 1996). It is therefore possible that the observed nuclear phenotype is related to mitotic stimulation and mitotic chromatin condensation; the distinct chromatin

clumps seen in overexpressing cells may thus have represented deformed chromosomes. After all, HMW FGF-2 is a very basic protein, and nuclear overload with a basic protein may be expected to have charge-dependent, in addition to specific, effects on the nuclear contents. To start addressing this, we followed a strategy previously used by other investigators to identify mitotic chromatin, namely staining with antibodies against markers such as phosphorylated-H1 or phosphorylated-H3, associated with mitotic condensation (Bradbury 1992; Chadee et al. 1995; Van Hooser et al. 1998; Hendzel et al. 1997; Wei et al. 1999). Phosphorylation of H3 is now considered to play an essential role in mitotic chromosome condensation (Van Hooser et al. 1998; Wei et al. 1999). Our data showed that neither H1 nor H3 were phosphorylated in HMW FGF-2 overexpressing cells, therefore the chromatin phenotype observed was not caused by some form of mitotic condensation.

4 Concluding Remarks

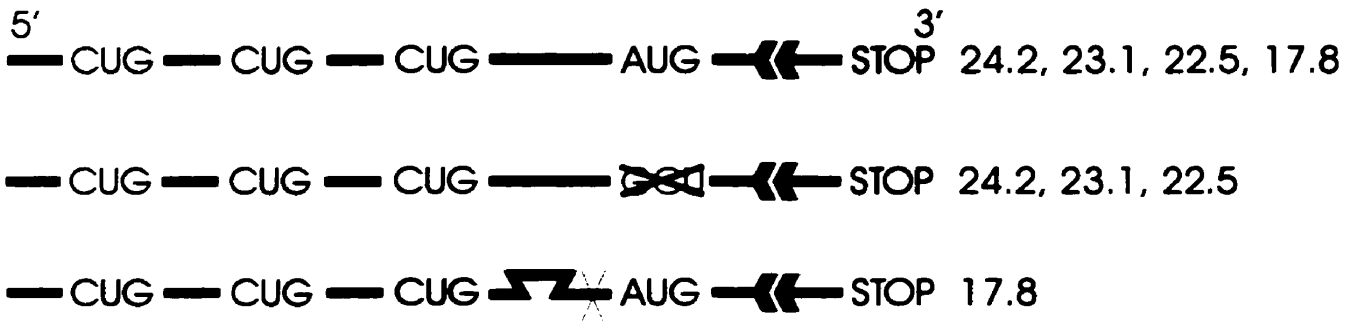
In conclusion, we have provided evidence indicating that the chromatin 'clumping' and separation caused by HMW FGF-2 is not related to the characterized forms of apoptosis, and it is not related to mitosis, but it represents a novel nuclear disruption phenotype. Although the basic charge of HMW FGF-2 may contribute to the observed chromatin phenotype, it is likely not the only factor responsible for the phenotype of clumping. Localization of LMW FGF-2 to the nucleus does not have the same clumping effects, even though LMW FGF-2 is also a very basic protein. It is probable that the N-terminal extension of HMW FGF-2 plays an important role in bringing about the

observed chromatin alterations by direct interaction with chromatin. This is currently studied in our laboratory, but it is beyond the scope of this thesis.

Several important questions remain to be addressed. Is the phenotype observed particular to cardiac myocytes or is it encountered in all cells? Preliminary evidence would suggest that the phenotype of nuclear clumping is not encountered in transfected cell lines, or primary fibroblasts. Cardiac myocytes are not highly proliferative cells, and they are destined to become apparently terminally differentiated. Their response to HMW FGF-2 may be related to their proliferative properties and their possible resistance to transformation. Is the phenotype dose-dependent, and/or cell and stage dependent? Levels of accumulation of HMW FGF-2 may be important in the manifestation of the 'clumped' phenotype. Transient transfection results in a fraction of cells having variable levels of HMW FGF-2; preliminary studies in our laboratory have shown that HMW FGF-2 displays a dose-dependent ability to promote chromatin condensation. What is the physiological relevance of our findings? At this point and since levels of HMW FGF-2 are regulated by hormones and stress, it is tempting to speculate that its distinct properties towards chromatin may contribute to distinct changes in gene expression associated with the above conditions.

Figure 1

PLASMIDS USED



Schematic representation of the original (wild-type) human FGF-2 cDNA and each mutant driven from it. The wild-type human FGF-2 cDNA was subcloned into the EcoR1 site in pcDNA3 vector, produce pcDNA3-14 plasmid construct (upper). The ATG in wild-type FGF-2 cDNA was changed to GCT resulted in the synthesis of only three HMW FGF-2, this mutant was also subcloned into the EcoR1 site in pcDNA3 vector and produce pcDNA3-363 plasmid construct (middle). An oligonucleotide inserted at a unique Apa 1 site (frame shift) resulted in the synthesis of only 18 kDa FGF-2; this mutant was subcloned into Xho 1 site in pcDNA3 vector and termed as pcDNA3-18 (bottom). pcDNA3-363 and pcDNA3-18, which produce human HMW FGF-2 and LMW FGF-2, respectively , were used in our studies. This schematic figure was adopted from Florkiewicz RZ et al. Basic fibroblast growth factor gene expression. In: the fibroblast growth factor family. Edited by Baird A & Klagsbrun M. Ann. N.Y. Acad. Sci. 638:109-126.

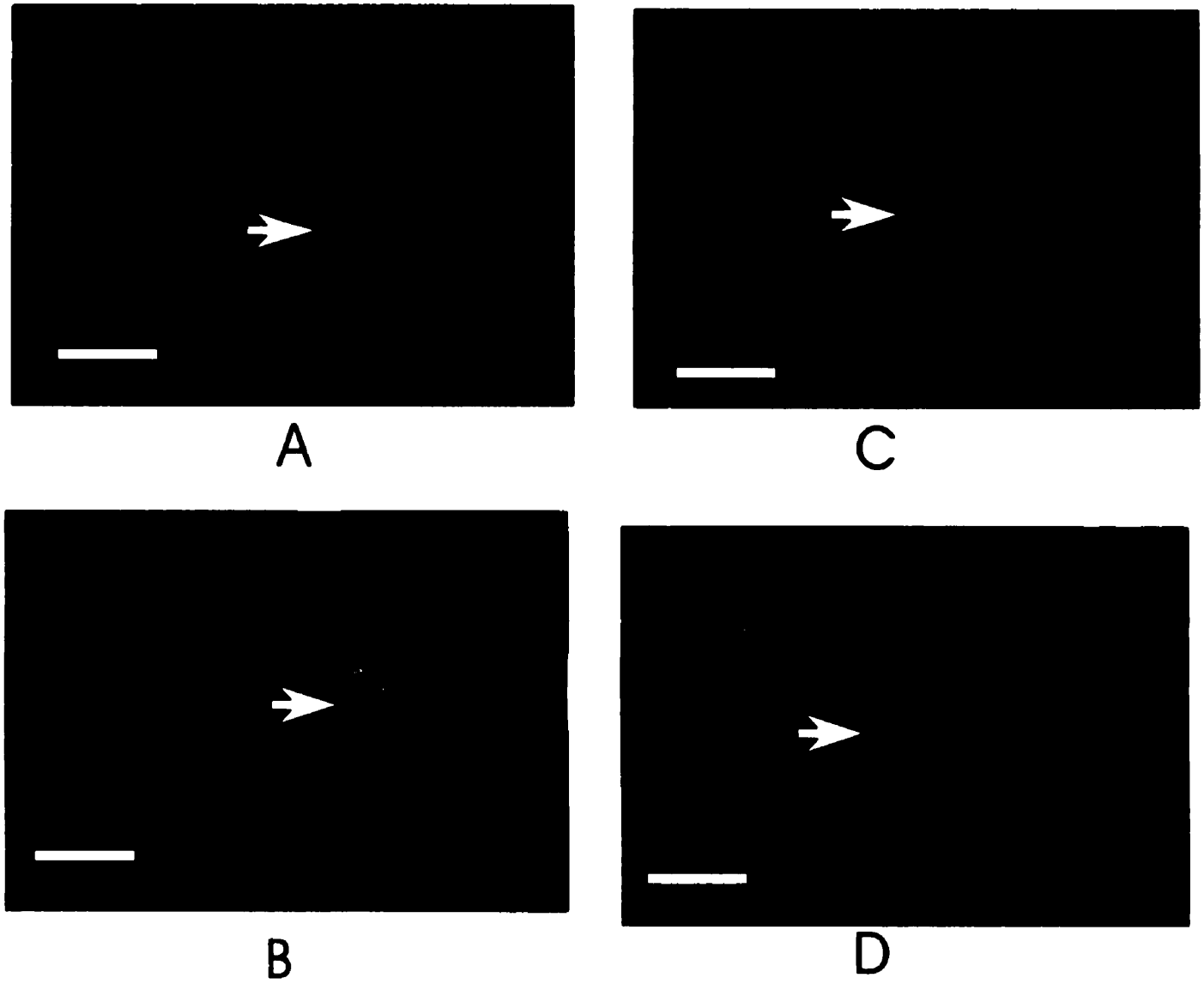


Figure 2

Figure 2

Human CUG-initiated HMW FGF-2, but not AUG-initiated LMW FGF-2, causes chromatin condensation and fragmentation in neonatal rat cardiac myocytes in culture.

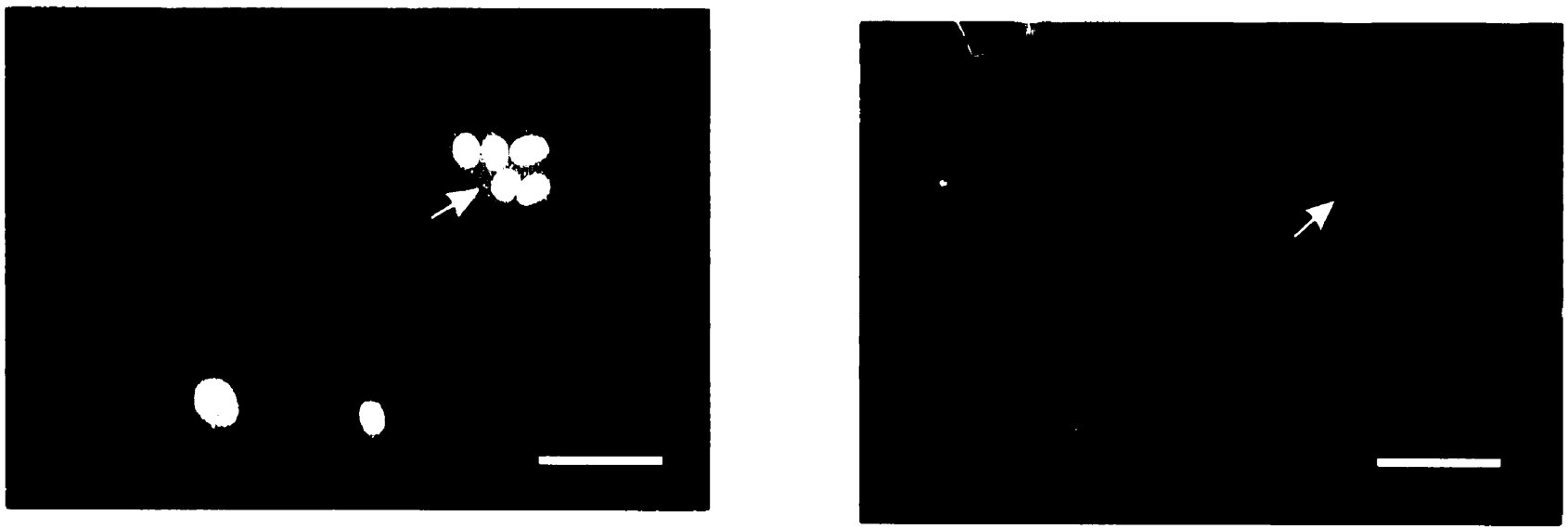
A & B: Myocytes transfected with HMW FGF-2. Double-fluorescence labeling for (A), FGF-2 (fluorescein), and (B) nuclear DNA (Hoechst 33342). Arrows indicate nuclei in overexpressing cells. Chromatin condensation and fragmentation is evident in the transfected cells. Bar=20 μm.

C & D: Myocytes transfected with LMW FGF-2. Double-fluorescence labeling for (C), FGF-2 (rhodamin), and (D) nuclear DNA (Hoechst 33342). Arrows indicate overexpressing cells. Chromatin condensation and fragmentation is not seen in the transfected cells. Bar=50 μm.

Figure 3

Overexpression of CUG-initiated FGF-2 is associated with multinucleation in rat cardiomyocytes.

A & B: Myocytes transfected with HMW FGF-2. Double-fluorescence labeling for (A), FGF-2 (fluorescein), and (B) nuclear DNA (Hoechst 33342). Arrows indicate presence of 5X nuclei in overexpressing cell. Bar=50 μm.



A

B

Figure 3

Figure 4

Bcl-2 does not prevent the effects of HMW FGF-2 on chromatin

A, B, C & D, E, F: Triple-fluorescence labeling for (A, D), FGF-2 (rhodamin), (B, E), Bcl-2 (fluorescein) and (C, F) nuclear DNA (Hoechst 33342). Arrows point to nuclei of cells overexpressing HMW FGF-2 and Bcl-2. Bar=20 μm.

Cardiac myocytes have been transfected simultaneously with HMW FGF-2 and Bcl-2. Nuclear condensation and fragmentation caused by FGF-2 can be observed in the presence of overexpression of Bcl-2.

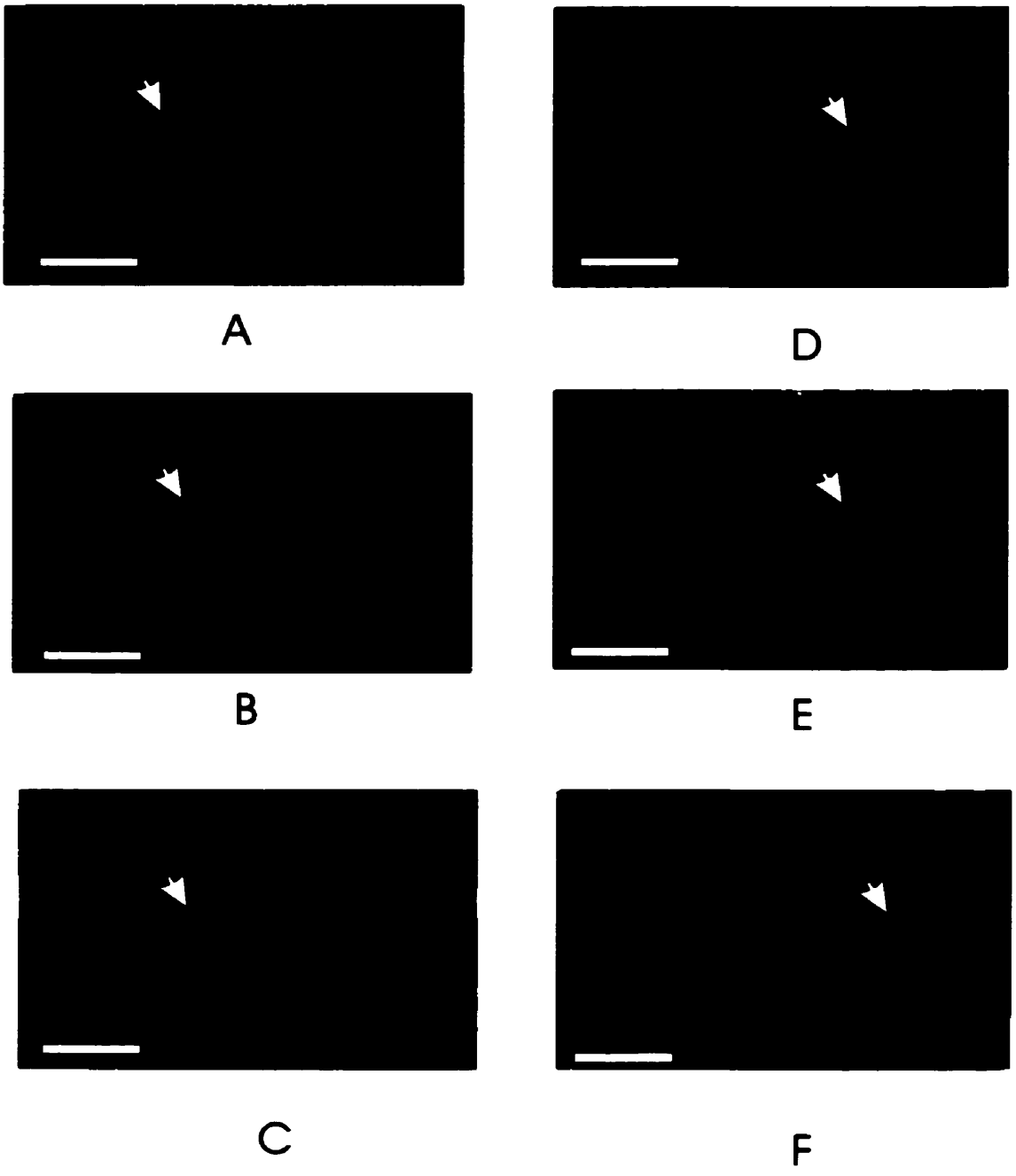


Figure 4

Figure 5

Apoptosis inhibitors (Caspase inhibitors, Bcl-2, and LMW FGF-2) do not prevent the effects of HMW FGF-2 on chromatin.

The percentage of cardiac myocyte DNA clumping refers to the number of HMW FGF-2 overexpressing cells with 'clumped' nuclei over the total number of HMW FGF-2 overexpressing cells. In the case of overexpression of both Bcl-2 and HMW FGF-2, we scored the number of myocytes overexpressing both Bcl-2 and HMW FGF-2 and presenting the 'clumped' phenotype, over the total number of myocytes overexpressing Bcl-2 and HMW FGF-2. Data were analyzed for statistical significance by a Student's t test; Error bar is SEM ($n=3$, $P<0.05$). Asterisk indicates the statistically significant difference from the HMW FGF-2 overexpressing cells.

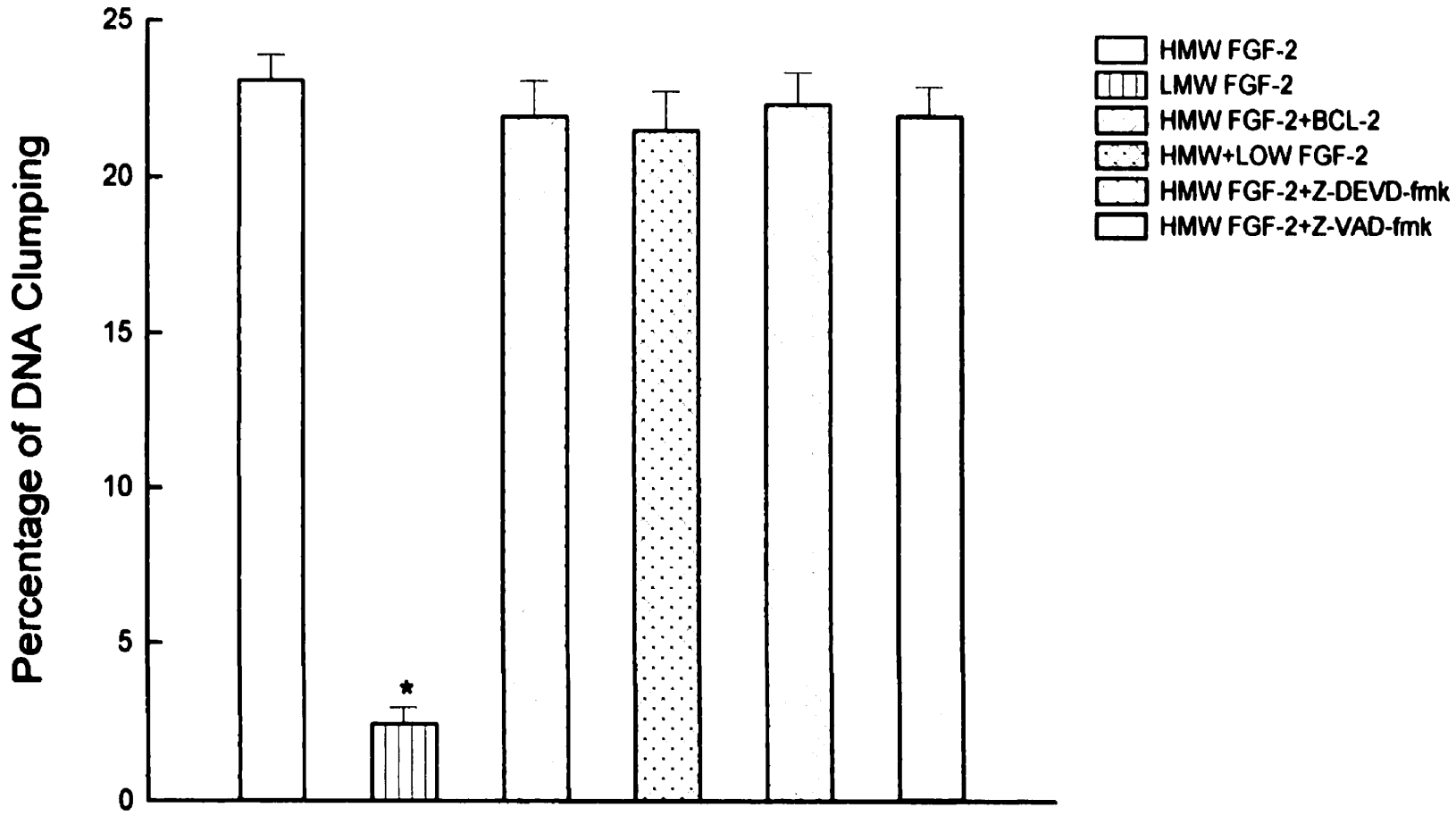

Percentage of DNA Clumping
25
20
15
10
5
0
*
HMW FGF-2
LMW FGF-2
HMW FGF-2+BCL-2
HMW+LOW FGF-2
HMW FGF-2+Z-DEVD-fmk
HMW FGF-2+Z-VAD-fmk
Treatments

Figure 5

Figure 6

Overexpression of HMW FGF-2 is not associated with an apoptotic pattern of DNA cleavage.

Electrophoretic analysis of genomic DNA from control, vector-transfected cells, HMW FGF-2-transfected cells, and staurosporine-treated cells (positive control for apoptotic phenotype), as indicated.

DNA from HMW FGF-2 transfected cultures (10% transfection efficiency) shows no evidence of apoptotic DNA. However, the relatively low transfection efficiency may be masking the presence of apoptotic pattern.

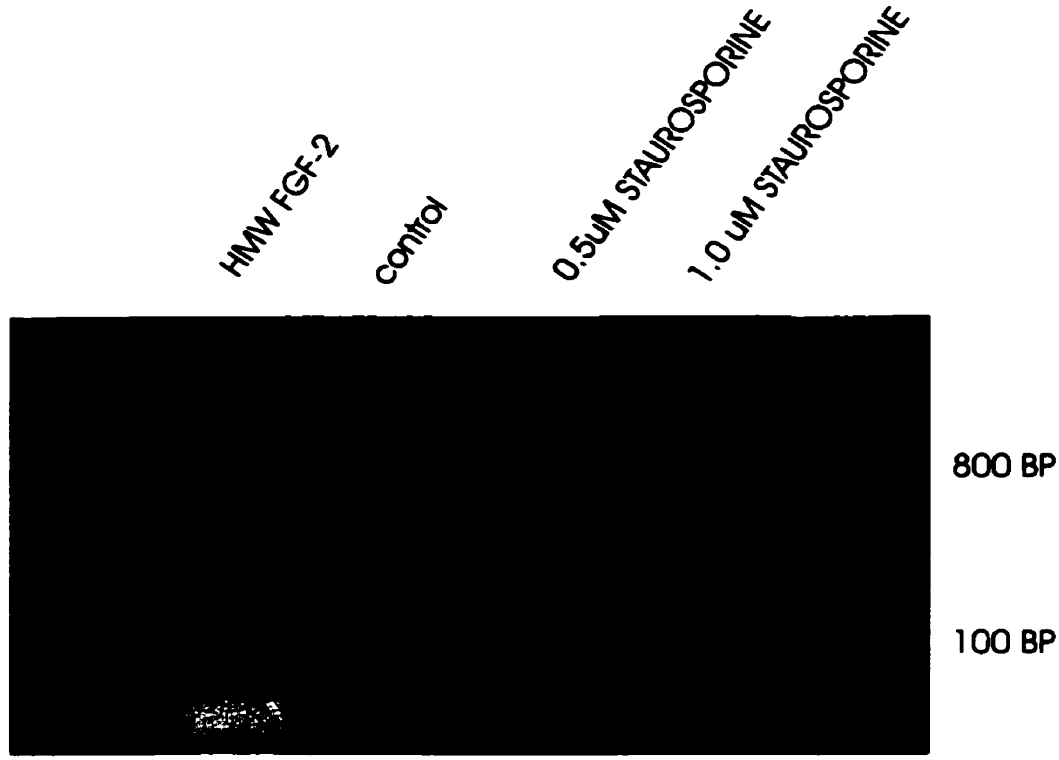

HMW FGF-2
control
0.5uM STAUROSPORINE
1.0 uM STAUROSPORINE
800 BP
100 BP

Figure 6

Figure 7

TUNEL staining and HMW FGF-2 induced nuclear phenotype

A, B, C & D, E, F: Triple-fluorescence labeling for (A, D), TUNEL, (B, E), FGF-2, and (C, F) nuclear DNA (Hoechst 33342). Bar=50 μm.

The majority (>80%) of myocytes overexpressing HMW FGF-2 and displaying condensed and fragmented chromatin stained negative for TUNEL (see A, B, C), suggesting that chromatin condensation caused by HMW FGF-2 does not require/induce an apoptosis-like mechanism. Arrows show cell overexpressing HMW FGF-2 and displaying condensed and fragmented chromatin had negative TUNEL staining.

BUT: A small percentage of myocytes overexpressing HMW FGF-2 and displaying condensed and fragmented chromatin stained for TUNEL (see D, E, F). It is suggested that this represent a non-HMW FGF-2-induced phenomenon since a similar degree of apoptosis is seen even in non-transfected cells. Arrows show cell overexpressing HMW FGF-2 and displaying condensed and fragmented chromatin had positive TUNEL staining.

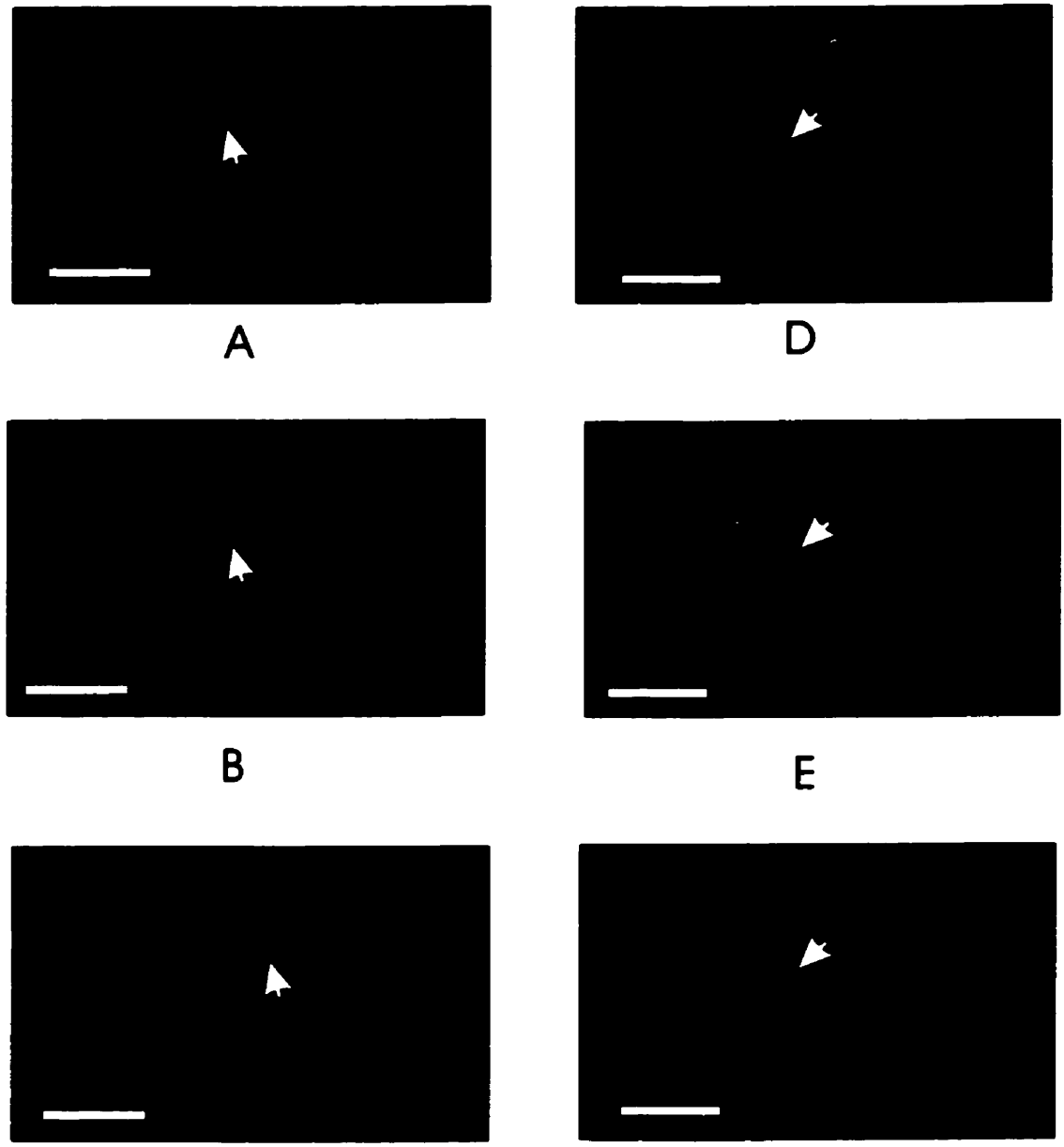


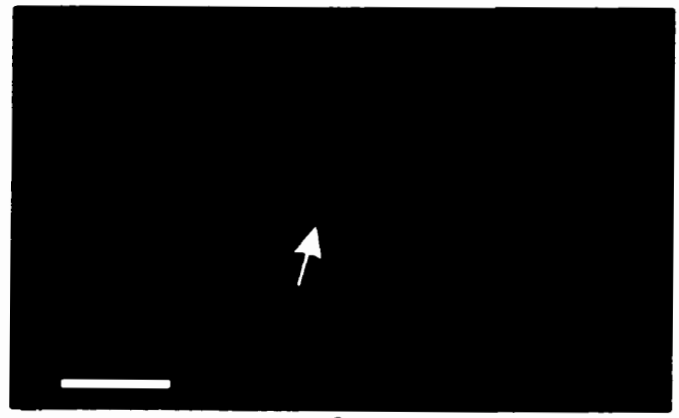
Figure 7

Figure 8

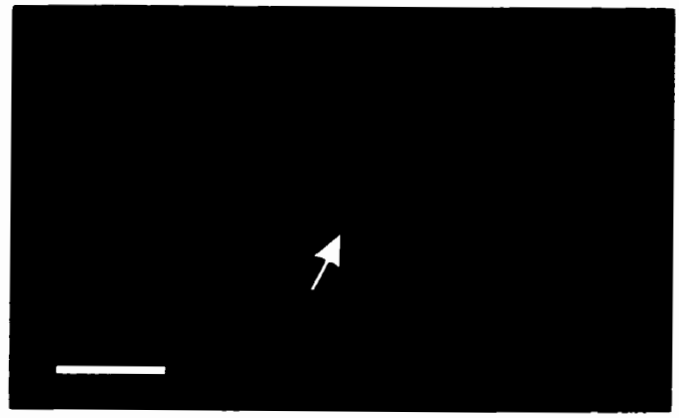
The nuclear lamina remains intact in cardiomyocytes overexpressing HMW FGF-2.

A, B & C: Triple-fluorescence labeling for, (A), Lamin-B, (B), FGF-2, (C), nuclear DNA. Bar=20 μm.

Apoptosis is associated with disruption of the nuclear lamina and degradation of lamins. The nuclear fragmentation however induced by HMW FGF-2 co-exists with an apparently intact nuclear lamina, as indicated by the nuclear anti-lamin B staining (arrow). Arrows show myocytes overexpressing HMW FGF-2 and displaying DNA clumping still had the intact nuclear lamina.



A



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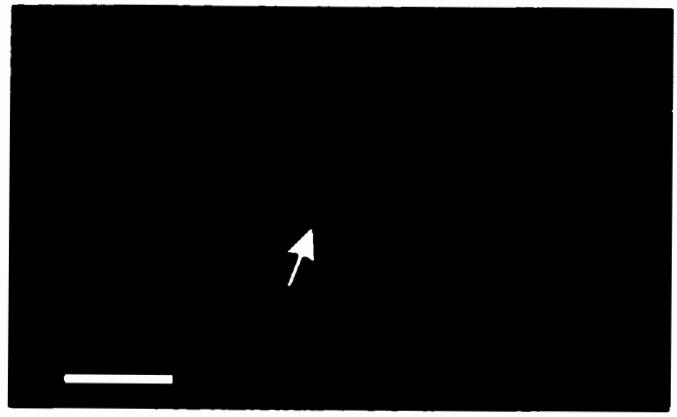


C

Figure 8

Figure 9

Histone H1 is not phosphorylated in the nuclei of myocytes overexpressing HMW FGF-2

A, B, C & D, E, F: Triple-fluorescence labeling for (A, D), phosphorylated histone H1, (B, E), FGF-2, and (C, F) nuclear DNA (Hoechst 33342). Bar=50 μm (A through C) and 20 μm (D through F).

Phosphorylation of H1 is associated with chromatin condensation required for mitosis. Antibodies to phosphorylated histone H1 therefore stain strongly nuclei that are about to enter, or are actively undergoing mitosis (indicated by arrowhead). These antibodies do not stain nuclei undergoing HMW FGF-2 induced chromatin condensation (indicated by arrow). The chromatin phenotype induced by HMW FGF-2 is not related to the onset of mitosis.

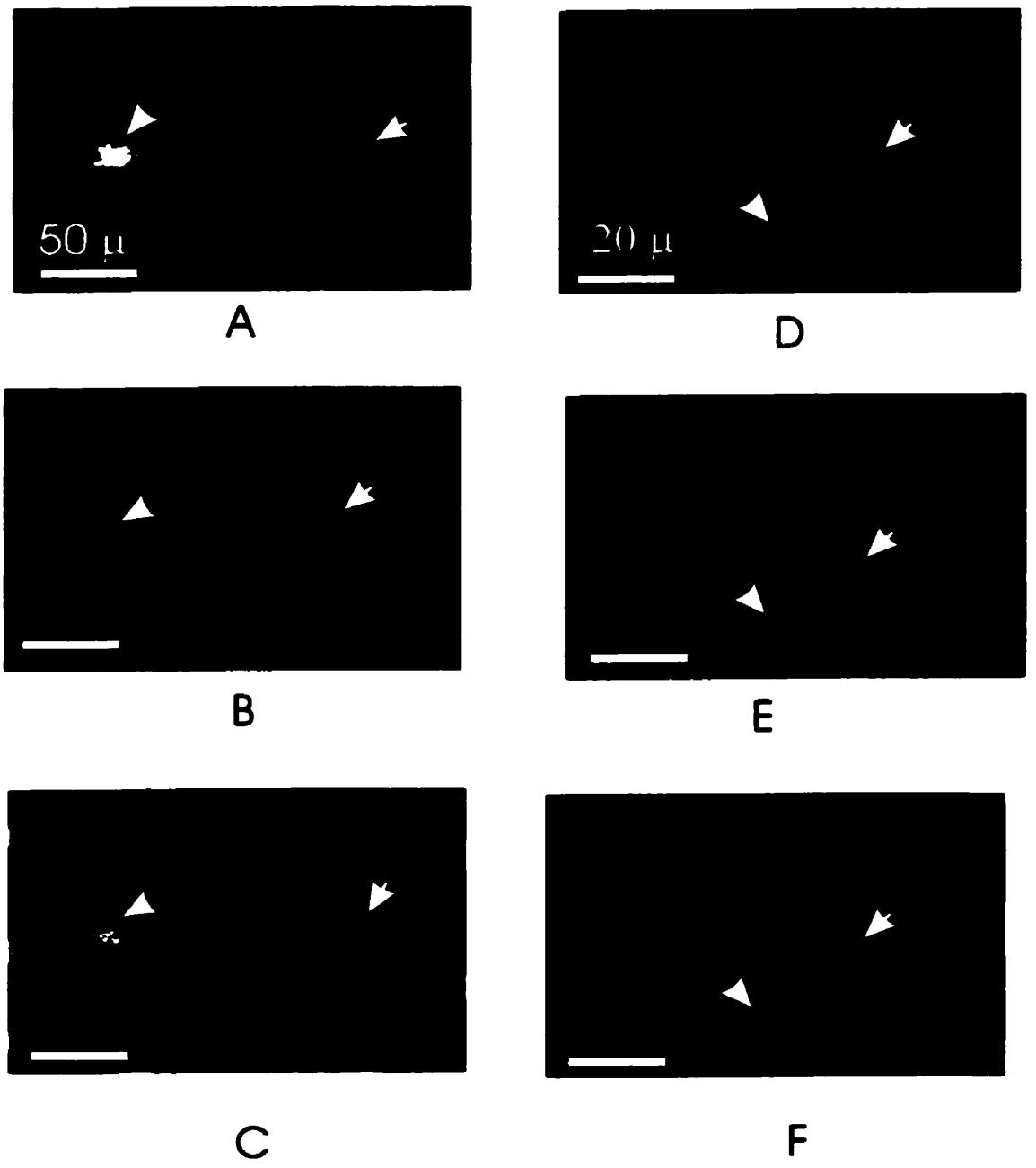


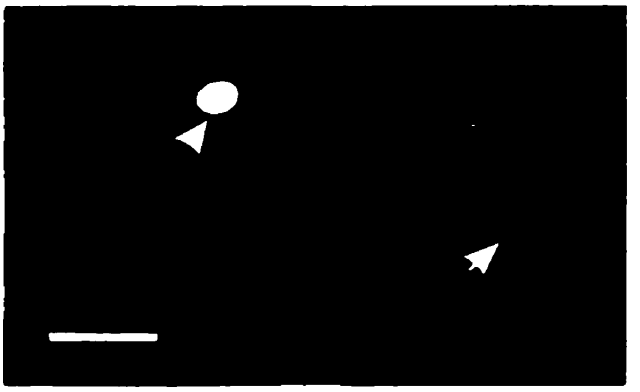
Figure 9

Figure 10

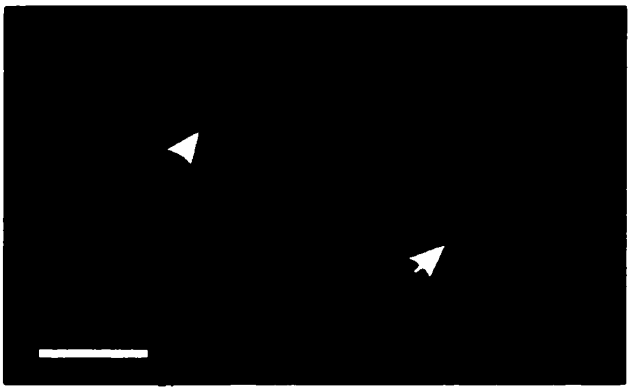
Histone H3 is not phosphorylated in the nuclei of myocytes overexpressing HMW FGF-2

A, B, C: Triple-fluorescence labeling for (A) phosphorylated histone H3, (B), FGF-2, and (C) nuclear DNA (Hoechst 33342). Bar=50 μm.

Phosphorylation of H3 is associated with chromatin condensation required for mitosis. Antibodies to phosphorylated histone H3 therefore stain strongly nuclei that are about to enter, or are actively undergoing mitosis (these nuclei are indicated by the arrowhead). These antibodies do not stain nuclei undergoing HMW FGF-2 induced chromatin condensation (indicated by the arrow). The chromatin phenotype induced by HMW FGF-2 is not related to the onset of mitosis.



A



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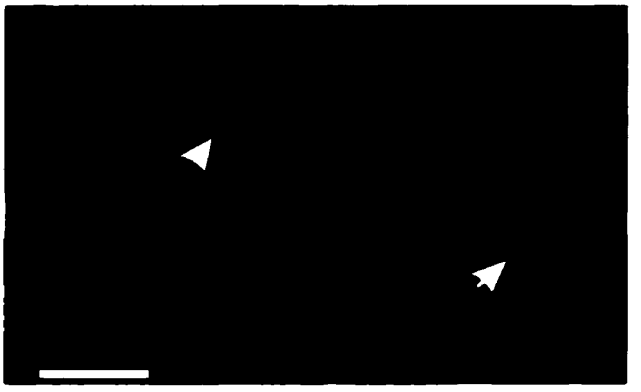

C

Figure 10

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