Apoptosis in myocardial ischaemia and infarction

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Recent studies indicate that, in addition to necrosis, apoptosis also plays a role in the process of tissue damage after myocardial infarction, which has pathological and therapeutic implications. This review article will discuss studies in which the role and mechanisms of apoptosis in myocardial infarction were analysed in vivo and in vitro in humans and in animals.

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T he common view on how cardiomyocytes die during or after myocardial infarction has altered in recent years. For a long time necrosis was regarded as the sole cause of cell death in myocardial infarction. Now, recent studies indicate that apoptosis also plays a role in the process of tissue damage subsequent to myocardial infarction. Although both necrosis and apoptosis result in the death of the cell, they differ in several morphological and cellular regulatory features.

Necrosis is characterised by the rapid loss of cellular homeostasis, rapid swelling as a result of the accumulation of water and electrolytes, early plasma membrane rupture, and the disruption of cellular organelles. As a result of the membrane rupture and subsequent leakage of a broad array of cellular material, necrosis induces an inflammatory response.[1–3]

Apoptosis or programmed cell death is, unlike necrosis, a highly regulated and energy requiring process. Apoptosis is characterised by shrinkage of the cell and the nucleus. The nuclear chromatin is condensed into sharply delineated masses, and eventually breaks up. The cell then detaches from the surrounding tissue. At this stage, extensions bud out from its membrane, which eventually seal off to form membrane enclosed vesicles, called apoptotic bodies, containing condensed cellular organelles and nuclear fragments. These apoptotic bodies are either rapidly phagocytosed by neighbouring cells or undergo degradation, which resembles necrosis in a process called secondary necrosis. However, apoptosis is generally considered not to trigger an inflammatory response.[4]

> "Although both necrosis and apoptosis result in the death of the cell, they differ in several morphological and cellular regulatory features"

However, recently there has been much debate concerning which morphological features accurately reflect apoptosis. Furthermore, it has been suggested that the process of apoptosis is regulated by a large variety of different cellular mechanisms. Therefore, Sloviter suggested that

perhaps the terms necrosis and apoptosis should be redefined as "passive cell death" and "active cell death", respectively.[5]

The fact that apoptosis plays a role in the tissue damage seen after myocardial infarction has pathological and therapeutic implications. Because apoptosis is a highly regulated process, a better understanding of the circumstances that specifically trigger apoptosis during and after myocardial infarction, and a better understanding of the cellular mechanisms that control apoptosis, could lead to therapeutic strategies to limit the amount of tissue damage in patients with myocardial infarction.

In this review article, we will discuss studies in which the role of apoptosis in myocardial infarction was analysed in vivo and in vitro. In these studies, apoptotic cells were detected using the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling method (TUNEL) combined with agarose gel DNA laddering, unless stated otherwise.

STUDIES IN HUMANS
Apoptosis in human cardiomyocytes after myocardial infarction

Studies in the ventricular myocytes of the hearts of patients who died of acute myocardial infarction (AMI) have shown that apoptosis plays a role in the process of cell death of cardiomyocytes. Saraste *et al* showed, in myocardial samples obtained from patients who died of acute AMI, that in addition to overt necrosis, a subset of myocytes undergoes apoptosis during ischaemia/reperfusion injury[6] (table 1). The apoptotic myocytes were most prominent in the border zones of recent infarction,[6–9] whereas very few apoptotic cells were present in the remote non-infarcted myocardium.[6] In line with this, increased uptake of Tc-99m labelled annexin V in the infarcted area, but not outside the infarcted

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Table 1 Overview of the prevalence and timing of apoptosis in the different models of investigation

Model	Prevalence of apoptosis	Source	Timing of apoptosis	Marker of apoptosis used
Human	Yes	Human	6–120 h after AMI	TUNEL + DNA laddering[6]
	Yes	Human	3–14 h after AMI	TUNEL + DNA laddering[8]
	Yes	Human	3.5–22 h after AMI	Annexin V[11]
In vivo animal	Yes	Rat	2 h to 7 d	TUNEL + Ab myosin heavy chain[12]
	Yes	Rat	2.25 h I	TUNEL ISEL + DNA laddering[13]
	Yes	Rat	45 min I and 1 h R	TUNEL ISEL + DNA laddering[14]
	Yes	Rat	24 h to 12 w	TUNEL[14]
	No	Dog	7 h I	TUNEL + DNA laddering[15]
	Yes	Dog	6 h I and 6 h R	TUNEL + DNA laddering[15]
	Yes	Rat	3 h I	TUNEL + DNA laddering[16]
	Yes	Rabbit	30 min and 4 h R	TUNEL + DNA laddering[17]
	No	Rat	10 min I and 3 h R	TUNEL + sandwich enzyme assay[18]
	Yes	Rat	20 min I and 3 h R	TUNEL + sandwich enzyme assay[18]
	Yes	Dog	30 min I and 3 h R	DNA laddering[19]
	Yes	Rat	30 min I and 3 h R	TUNEL + DNA laddering[20]
	Yes	Rat	30 min I and 3 h R	Elisa and westernblot[21]
	Yes	Rat	30 min I and 6 h R	TUNEL + DNA laddering[22]
	Yes	Mouse	1 h – 72 h	TUNEL + DNA laddering[23]
	Yes	Rat	until 24 h	TUNEL + DNA laddering[24]
	No	Rabbit	4,5 h I	TUNEL + DNA laddering[25]
	Yes	Rabbit	30 min I and 4 h R	TUNEL + DNA laddering[25]
	Yes	Mouse	15 min I and 30 min R	Annexin V and DNA laddering[26]
Isolated animal hearts	Yes	Rat/Mouse	25 min I and 4 h R	TUNEL[27]
	Yes	Rat	30 min I and 2 h R	TUNEL + DNA laddering[28]
	Yes	Rat	30 min I and 2 h R	TUNEL + DNA laddering[29]
	No	Rat	60 min I	TUNEL + Ab myosin heavy chain[30]
	Yes	Rat	15 min I and 2 h R	TUNEL + Ab myosin heavy chain[30]
	Yes	Rabbit	30 min I and 1 h R	TUNEL + DNA laddering[31]
Isolated cardiomyocytes	Yes	Rat	48 h I	TUNEL + DNA laddering[32]
	Yes	Rat	48 h I and 3 h R	TUNEL + DNA laddering[32]
	No	Rat	18 h I and 12 h R	Annexin V/PI + TUNEL + DNA laddering[33]
	Yes	Rabbit	30 min I and 24 h R	TUNEL[34]
	No	Rat	3 h I and 2 h R	Annexin V/PI[35]
	No	Mouse	24 h I	DNA laddering[36]
	Yes	Mouse	24 h I and 8 h R	DNA laddering[36]

AMI, acute myocardial infarction; d, days; h, hours; I, ischaemia; min, minutes; PI, propidium iodide; R, reperfusion; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labelling; w, weeks.

area in patients with AMI, suggests the occurrence of apoptosis in this area,[6] although it should be noted that annexin V stains necrotic cells in addition to apoptotic cells. In contrast, other studies have shown a substantial amount of apoptosis in the "remote from ischaemia" areas also.[8] [10]

Bcl-2 and Bax
As mentioned earlier, apoptosis is a highly regulated process in which several regulatory proteins play a part, and in which the balance between an array of regulatory proteins decides the fate of the cell. The expression of two such regulatory proteins, bcl-2 and Bax, has been studied in the hearts of patients who died of AMI.[37] Bcl-2, an apoptosis inhibitor, is not expressed in non-infarcted myocardial tissue,[38] but it is expressed in cardiomyocytes surrounding infarcted areas soon after the onset of infarction. However, no bcl-2 was found in the infarcted area itself. Bax is a member of the bcl-2 family and, when overexpressed, accelerates apoptosis. It also counters the death repressor activity of bcl-2.[39] In contrast to bcl-2, Bax has a low basal expression in human hearts without cardiac disease.[37] The overexpression of Bax was found in two of 15 hearts at the acute stage of infarction (between six hours and 20 days after the onset of myocardial infarction (MI)). At the old stage (more than a month after the onset of MI) Bax overexpression was found in 10 of 12 hearts, predominantly in the areas surrounding infarcted areas. In these areas the expression of bcl-2, present in the acute stage, had disappeared. Therefore, the Bax to bcl-2 ratio is high and may be related to apoptotic cell death in these areas in the old infarction.[37]

Fas
In AMI, soluble Fas (sFas: an inhibitor of apoptosis) values increase, whereas sFas ligand (an inducer of apoptosis) values do not change.[40] Circulating sFas values are significantly higher in acute MI than in old MI. Remarkably, sFas values increase in AMI independent of infarct size.

IN VIVO ANIMAL MODELS
Apoptosis triggered by ischaemia and/or reperfusion?
Cell death resulting from MI has been extensively investigated in vivo in various animal models. Usually, MI was induced by the occlusion of a major coronary artery.

Upon permanent occlusion of a coronary vessel in rats, apoptosis occurred in the ischaemic region, the area immediately bordering the ischaemic region and in the remote from ischaemia region. Therefore, it was suggested that apoptosis is the major determinant of infarct size. Necrosis occurred less often and was seen only in the ischaemic region.[12] In a similar experiment in rats, Fliss and Gattinger also showed that apoptosis occurred after constant ischaemia.[13] However, they found that apoptosis appeared solely in the ischaemic myocardium, not in the bordering or remote from ischaemia regions. Although reperfusion after 45 minutes of ischaemia appeared to attenuate apoptosis in the infarcted area, it enhanced apoptosis in the bordering zones and the remote from ischaemic regions. In a similar study in rats by Palojoki et al,[14] enhanced apoptosis was seen from 24 hours up to 12 weeks after experimental MI in the ischaemic area and in its

border zone. Apoptosis was also increased in the remote non-infarcted tissue up to four weeks after infarction.[14] However, in this study apoptosis was measured by the TUNEL method only.

> "A better understanding of the cellular mechanisms that control apoptosis could lead to therapeutic strategies to limit the amount of tissue damage in patients with myocardial infarction"

Apoptosis after ischaemia/reperfusion was also shown in an in vivo dog model in the ischaemic area.[15] However, apoptosis was not found in this area in dogs subjected to ischaemia alone.

Bcl-2 and Bax

In an in vivo experiment in rats, DNA strand breaks were seen as early as three hours after coronary artery occlusion.[41] Apoptosis was accompanied by a decrease in bcl-2 protein values and an increase in the expression of Bax, indicating a role for these proteins in the regulation of ischaemia induced apoptosis in rats. In another in vivo rat model it was found that EAT/mcl-1, a bcl-2 related immediate early gene, is strongly upregulated as early as 30 minutes after coronary artery occlusion.[16] In line with this, in transgenic mice overexpressing human bcl-2, myocardial reperfusion injury was reduced.[42] This reduction of reperfusion injury correlated with a significant reduction in apoptosis, as measured by means of the TUNEL staining method only. In dogs, reperfusion resulted in reduced bcl-2 expression and increased Bax expression in the ischaemic area, whereas after permanent ischaemia, bcl-2 and Bax values did not change.[15]

Fas

In an in vivo rabbit model it was shown that Fas receptor expression was dramatically upregulated in the border zone of histologically infarcted myocardium after ischaemia/reperfusion.[17] In line with this, carvedilol, a vasodilating β adrenoceptor antagonist and a potent antioxidant, caused a 77% reduction of apoptotic cells in the border zone and at the same time significantly reduced Fas receptor expression after ischaemia/reperfusion.[17] In addition, in an in vivo rat model it was found that Fas receptor expression increased 131 fold in cardiomyocytes after coronary artery occlusion.[43] These results indicate that the Fas receptor is involved in ischaemia/reperfusion induced apoptosis in rabbit and rat hearts.

Preconditioning

Ischaemic preconditioning is a phenomenon whereby a series of brief periods of alternating ischaemia and reperfusion increase myocardial tolerance to the subsequent prolonged ischaemia. This phenomenon was first described by Murry *et al*.[44] Since then, numerous studies have shown the cardioprotective effect of preconditioning in different animal species and in humans.[45–57]

Piot *et al* showed that ischaemic preconditioning in rats caused a significant reduction in the amount of apoptosis and infarct size.[18] However, the exact mechanisms by which ischaemic preconditioning causes this cardioprotective effect are still unclear. A correlative effect of reduced myocardial apoptosis, reduced Bax expression, increased bcl-2 expression, and reduced neutrophil accumulation in ischaemic preconditioning experiments in rats and dogs has been suggested.[19 20] Ischaemic preconditioning also seems to attenuate the ischaemia/reperfusion induced activity of caspase 1 and caspase 3 in rats.[21]

Accumulative evidence points to a role for protein kinase C (PKC) as a final common pathway in the cardioprotective effect of ischaemic preconditioning in rats. It was shown that pretreatment with calphostin C, an inhibitor of PKC, completely aborted the protective effect of ischaemic preconditioning on infarct size[58] and most apoptosis[22] caused by ischaemia/reperfusion. Piot *et al* suggest that in ischaemic preconditioning possible end effectors of PKC are ATP sensitive potassium channels, because it was shown that the activation of mitochondrial K_{ATP} channels induces preconditioning against MI and reduces ischaemic/reperfusion induced apoptosis through the PKC signalling pathway.[59]

Reactive oxygen species

Increased oxidative stress was found to coexist with apoptosis in the remote non-infarcted rat myocardium after myocardial infarction.[60] Longterm treatment with the antioxidants probucal and pyrrolidine dithiocarbamate, starting three days after MI in an in vivo rat ischaemia/reperfusion model, attenuated oxidative stress, myocyte apoptosis, caspase 3 activity, and the expression of p53, Bax, and caspase 3 protein in the remote non-infarcted myocardium.[61] These findings indicate a causal relation between oxidative stress and apoptosis in the remote non-infarcted rat myocardium after MI.

p53

One hypothesised general molecular mediator of hypoxia induced apoptosis is the tumour suppressor transcription factor p53. It is suggested that p53 may induce apoptosis by stimulating the expression of Bax and/or repression of bcl-2 expression.[62] In contrast, in an in vivo model of apoptosis in wild-type mice and homozygous p53 knockout mice, it was found that ischaemia induced apoptosis occurred as readily in the p53 knockout mice as in wild-type mice.[23] This finding indicates the existence in the heart of other, p53 independent, mechanisms of postischaemic apoptosis.

JAK/STAT pathway

In general, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is reported to be involved in the transduction of cytoprotective signals in stressful conditions, such as hypoxia.[63 64] In an in vivo rat experiment, it was found that rats treated with a specific JAK-2 inhibitor AG-490, subsequent to ischaemia, did show a significant upregulation of caspase 3 activity and Bax expression, in addition to a significant increase in the number of apoptotic cells compared with control rats.[65] These results indicate an antiapoptotic role for the JAK/STAT signalling pathway in the rat myocardium.

Stress activated protein kinases

The stress activated protein kinase (SAPK) signalling pathway has recently been implicated as an important mediator of apoptosis.[66] SAPK activity is dramatically increased in rabbit hearts after ischaemia/reperfusion.[17] Carvedilol, a β adrenoceptor antagonist, when administered before reperfusion, significantly reduces apoptosis and significantly attenuates SAPK activity in rabbit hearts.[17]

Other stress activated mitogen activated protein kinases (MAPKs) have also been implicated in ischaemia/reperfusion induced apoptosis. p38 MAPK activity was induced in the rat heart as early as 15 minutes after coronary artery occlusion and also by reperfusion.[67] Jun N-terminal kinase (JNK) activity was also induced after ischaemia followed by reperfusion in the rat heart, although no JNK activity was seen as a result of ischaemia alone. Both MAPK and JNK are suggested to play a role in apoptosis because the time course of the activation patterns of these kinases and the increase in DNA laddering are highly correlated.[67]

Insulin-like growth factor I

Insulin-like growth factor I (IGF-I) appeared to be cardioprotective in a rat in vivo ischaemia/reperfusion model.[68] When administered one hour before ischaemia, IGF-I attenuated

polymorphonuclear neutrophil accumulation in the ischaemic area. Furthermore, IGF-I significantly attenuated the incidence of myocyte apoptosis after myocardial ischaemia and reperfusion.[68] In addition, in transgenic mice overexpressing human IGF-I, it was found that both apoptosis and necrosis were attenuated in the viable myocardium after infarction.[69]

> "Insulin-like growth factor I appeared to be cardioprotective in a rat in vivo ischaemia/reperfusion model"

Interestingly, IGF-I also promotes the release of physiological amounts of nitric oxide (NO).[70 71] In an in vivo mouse model it was shown that the inhibition of endogenous NO synthesis increased apoptosis during ischaemia.[72] It seems that endogenous NO suppresses apoptosis by interfering with the caspase cascade, because the inhibition of endogenous NO correlated with increased caspase activity, whereas bcl-2 and Bax protein values were unchanged.[72] However, it cannot be excluded that reduced apoptosis is a systemic effect rather than an effect of endogenous NO on the caspase machinery because NO, through an effect on microcirculation, influences perfusion.

Heat shock protein
Heat shock protein 70 (HSP70) is a self preservation protein that maintains cellular homeostasis under environmental stress. HSP70 acts as a molecular chaperone and plays an important role in protein folding, assembly, transport, and degradation although it also helps to prevent protein denaturation and aggregation.[73] In an in vivo experiment in rats it was shown that overexpression of HSP70, via gene transfection, reduced apoptosis after ischaemia/reperfusion.[74]

Calcineurin
In rats adenovirally transfected with constitutively active calcineurin cDNA, ischaemia/reperfusion induced apoptosis was significantly attenuated, whereas the inhibition of endogenous calcineurin reversed the antiapoptotic effects.[75] These results indicate a calcineurin dependent protection from apoptosis in cardiomyocytes.

Tumour necrosis factor
Recently it was found that tumour necrosis factor (TNF) is expressed within the myocardium of rats[76] and dogs[77] in response to ischaemic injury.[78] TNF has dual effects with regard to apoptosis. It provokes the expression of several antiapoptotic factors in a variety of mammalian cell types, although it can also induce apoptosis in mouse fibroblasts.[79] In an in vivo ischaemia model in mice lacking none, one, or both types of TNF receptor, it was found that endogenous TNF protected cardiomyocytes against ischaemia induced apoptosis.[80]

Caspases
In general, caspases form a key step in the process of apoptosis. In a rat and rabbit in vivo ischaemia/reperfusion model, the tripeptide inhibitor of the caspase interleukin 1β converting enzyme (ICE) family of cysteine proteases—ZVAD-fmk—was able to reduce infarct size and attenuated the amount of TUNEL positive cardiomyocytes.[24] Remarkably, ZVAD-fmk might also interfere with myocardial necrotic cell death because it was found that ICE-like inhibitors attenuate both apoptotic and necrotic cell death in an in vitro system of chemical hypoxia.[81 82] Therefore, the reduction in infarct size might result from both reduced apoptosis and necrosis. In a similar model, YVAD-aldehyde, a caspase 1-like protease inhibitor and DEVD-aldehyde, a caspase 3-like protease inhibitor, both attenuated the amount of myocardial apoptosis.[83] Remarkably however, these two inhibitors did not reduce infarct size.

Overexpression of cardiac specific caspase 3 in transgenic mice induced transient depression of cardiac function, caused abnormal ultrastructural damage to the nucleus in addition to the mitochondrial and myofibrillar content, and increased infarct size. However, it did not induce an apoptotic response that progressed through to the late apoptotic phases, such as the production of apoptotic bodies.[84]

Kallikrein
The activity of kallikrein leads to the production of intact kinins, which act through specific receptors to activate second messengers to trigger biological effects such as vasodilation and vasoconstriction.[85] The tissue kallikrein–kinin system components have been identified in the heart.[86–88] In an in vivo rat experiment it was found that, through the delivery of the human tissue kallikrein gene, myocardial infarction and apoptosis were significantly reduced after ischaemia/reperfusion injury.[85] This effect of kallikrein is probably mediated through an NO–cGMP dependent signal transduction pathway.[85]

Influence of age on apoptosis
In a mouse in vivo ischaemia/reperfusion model it was found that there are age associated differences in reperfusion injury between young (6–8 months) and old (22–24 months) mice.[89] It was shown that mortality was higher and infarct size was significantly larger in old animals, compared with young animals. Furthermore, the occurrence of DNA fragmentation was significantly higher in old hearts, indicating that the amount of apoptosis as a result of ischaemia/reperfusion is greater in old hearts.[89] A higher occurrence of DNA fragmentation was also seen in an in vivo rat model.[90] In this model, DNA fragmentation began and peaked earlier after infarction in the old heart than in the young heart, and bcl-2 and Bax showed a heightened baseline expression in the old hearts, which might indicate a higher degree of chronic stress in the old hearts.[90] The hearts of older animals might therefore be more susceptible to ischaemia/reperfusion related apoptosis.

ISOLATED ANIMAL HEARTS
The studies described below with isolated hearts were carried out in hearts that were perfused with a perfusion buffer (usually a Krebs Heinselheit buffer) in an atmosphere of 95% O_2 and 5% CO_2 at 37°C.

Fas
In isolated rat and mouse hearts subjected to ischaemia/reperfusion, it was found that soluble Fas ligand was released after the onset of reperfusion.[27] Isolated mouse hearts lacking functional Fas show a significant reduction in cell death after ischaemia/reperfusion, compared with wild-type mice, suggesting a proapoptotic role.[27]

Preconditioning
In isolated rat hearts, it has also been shown that preconditioning reduces cardiomyocyte apoptosis and oxidative stress.[28 91] Preconditioning in this model induced tyrosine kinase phosphorylation, leading to the activation of MAPKAP kinase 2 and p38 MAPK, and was associated with induced expression of bcl-2 mRNA and translocation and activation of the nuclear transcription factor nuclear factor κB (NfκB).[91] AP-1, another transcription factor, which is highly upregulated by prolonged ischaemia followed by reperfusion, is unaffected by preconditioning.[28] Preincubation with DMTU, a hydroxyl radical scavenger, or SN50, an NFκB blocker, abolished the antiapoptotic effect of preconditioning, and this is possibly related to the prevention of the preconditioning induced upregulation of bcl-2.[91] Indeed, in rat hearts treated with antisense bcl-2 oligonucleotides, the antiapoptotic effects

of preconditioning were abolished, as was the reduced oxidative stress, suggesting an antioxidant role for bcl-2 in preconditioning.[92]

Reactive oxygen species

In isolated perfused rat hearts it was shown that apoptosis only occurred in reperfused hearts when oxidative stress was induced.[29] The reperfusion injury effects could be reversed by epselen, which removed the oxidative stress from the heart, indicating that oxidative stress in this model plays a role in the development of ischaemia/reperfusion related apoptosis.[29]

p53

In contrast to animal in vivo studies, in isolated rat hearts, mRNA encoding the proapoptotic oncogene p53 was highly induced by reperfusion, whereas it was barely detected after ischaemia alone.[30] Ischaemic preconditioning prevented the activation of p53 and inhibited apoptosis.[30]

JAK/STAT pathway

Isolated rat hearts subjected to ischaemia/reperfusion show activation of STAT5A and STAT6. Treatment of these hearts with tyrphostin AG490, which inhibits JAK2 phosphorylation, resulted in reduced infarct size and apoptosis.[93]

Stress activated mitogen activated protein kinases

In isolated rat hearts, SAPK activity is raised as a result of ischaemia/reperfusion.[94] Treatment of these hearts with magnesium tanshinoate B, a bioactive compound isolated from Danshen, resulted in a reduction of apoptotic nuclei. Furthermore, it abolished the raised SAPK activity, suggesting a proapoptotic role for SAPK in ischaemia/reperfusion induced apoptosis in rats.[94]

> "In addition, p38 MAPK and JNK activities were greatly increased after ischaemia/reperfusion in an isolated rat heart model"

It has also been found that myocardial ischaemia/reperfusion results in the activation of p38 MAPK, whereby reperfusion has a more profound effect on p38 MAPK activity than ischaemia alone.[31] [67] [95] [96] In line with this, in isolated rabbit hearts the p38 MAPK inhibitor SB 203580 greatly reduced postischaemic myocardial apoptosis and significantly improved cardiac function recovery after reperfusion.[31] In addition, p38 MAPK and JNK activities were greatly increased after ischaemia/reperfusion in an isolated rat heart model.[97] However, inhibition of the extracellular signal related kinase (ERK) significantly increased the number of apoptotic cells, suggesting that p38 MAPK and JNK mediate myocardial apoptosis and that ERK plays a protective role.[97]

Heat shock protein

Recent reports indicate a role for the small HSP family in ischaemic preconditioning.[98] [99] αB crystalline is a member of the small HSP family and is the most abundantly expressed stress protein in the heart.[100] In transgenic mice overexpressing αB crystalline, it was found that the overexpression of αB crystalline results in attenuated apoptotic myocardial cell death, decreased oxidative stress, and a decrease in the extent of infarction, upon ischaemia/reperfusion.[101]

Caspases

In isolated rat hearts subjected to prolonged ischaemia, a significant accumulation of cytochrome c occurred in the cytosol, which was accompanied by activation of caspase 3-like proteases.[102] In contrast, caspase 1-like proteases were not activated.

ISOLATED AND CULTURED CARDIOMYOCYTES

Apoptosis induced by hypoxia?

Exposure of cultured neonatal rat cardiomyocytes to chronic hypoxia causes the activation of the terminal apoptotic machinery, including cytochrome c release, effector caspase activation, and cleavage of the death substrate poly(ADP-ribose) polymerase (PARP).[32] [103] The same effects were seen after exposure of neonatal rat cardiomyocytes to metabolic inhibition using 2-deoxyglucose.[104] These results indicate that both hypoxia and metabolic inhibition induce apoptosis through the mitochondrial pathway. Reoxygenation further increased the number of apoptotic cells and resulted in a decrease in bcl-2 and an increase in the Fas receptor.[32] Interestingly, these effects could be countered by incubation with either recombinant transforming growth factor β1 (TGF-β1) or aggregated platelet supernatant.[32] Aggregated platelet supernatant was found to protect the heart from reperfusion induced apoptosis, partly because of platelet released TGF-β1.[32] In contrast, in isolated ventricular rat cardiomyocytes, 60 minutes of anoxia alone did not increase annexin V staining, but induced a significant increase in propidium iodide staining.[105] An increase in annexin V staining was seen only after a period of reperfusion. In contrast, in another study with rat cardiomyocytes anoxia/reoxygenation caused necrosis in isolated rat cardiomyocytes but failed to induce apoptosis.[33]

Fas

In an in vitro study using neonatal rat cardiomyocytes it was shown that the expression of mRNA for the Fas antigen was upregulated twofold under hypoxic conditions, whereby Fas receptor and Fas ligand induction correlated with induced apoptosis.[106] Recently, it was also shown that STAT1 induced Fas receptor and Fas ligand expression after ischaemia/reperfusion in neonatal rat cardiomyocytes.[107] Furthermore, in primary adult rat culture, ischaemia followed by reperfusion caused a pronounced increase in sensitivity to the apoptotic effects of soluble Fas ligand.[27]

Preconditioning

In isolated adult cardiomyocytes from rabbit hearts, ischaemic preconditioning attenuated cardiomyocyte apoptosis, which correlated with a diminution in the fall of intracellular pH.[34] The antiapoptotic effects of ischaemic preconditioning could be prevented by blocking the vacuolar proton ATPase, suggesting that the maintenance of intracellular pH homeostasis attenuates apoptosis in ischaemic preconditioning.[34]

Reactive oxygen species

Oxidative stress through H_2O_2 and the NO donor, N_2-acetyl-S-nitroso-DL-penicillinaminamide (SNAP), induced apoptosis in ventricular cardiomyocytes isolated from a rat heart.[35] In isolated rat cardiomyocytes, a correlation between the apoptotic effect of SNAP or YC-1 (a direct activator of soluble guanylyl cyclase) and the increased activity of soluble guanylyl cyclase (that is, the intracellular cGMP content) was seen.[35] It has been suggested that NO induces apoptosis in a cGMP dependent manner and necrosis in a cGMP independent manner.

Both reactive oxygen species H_2O_2 and O_2^- induced apoptosis in neonatal rat cardiomyocytes, which was associated with an increase in p53 protein content, whereas protein concentrations of bcl-2 and Bax were unaltered.[108] Interestingly, both H_2O_2 and O_2^- induced apoptosis through distinct pathways. H_2O_2 induced the release of cytochrome c, activation of CPP32, and cleavage of PARP, through Bax and Bad (another proapoptotic bcl-2 family member). This can be suppressed by the pharmacological opening of the mitochondrial ATP sensitive K^+ channels by diazoxide, which preserves mitochondrial integrity.[109] In contrast, O_2^- promoted apoptosis through the
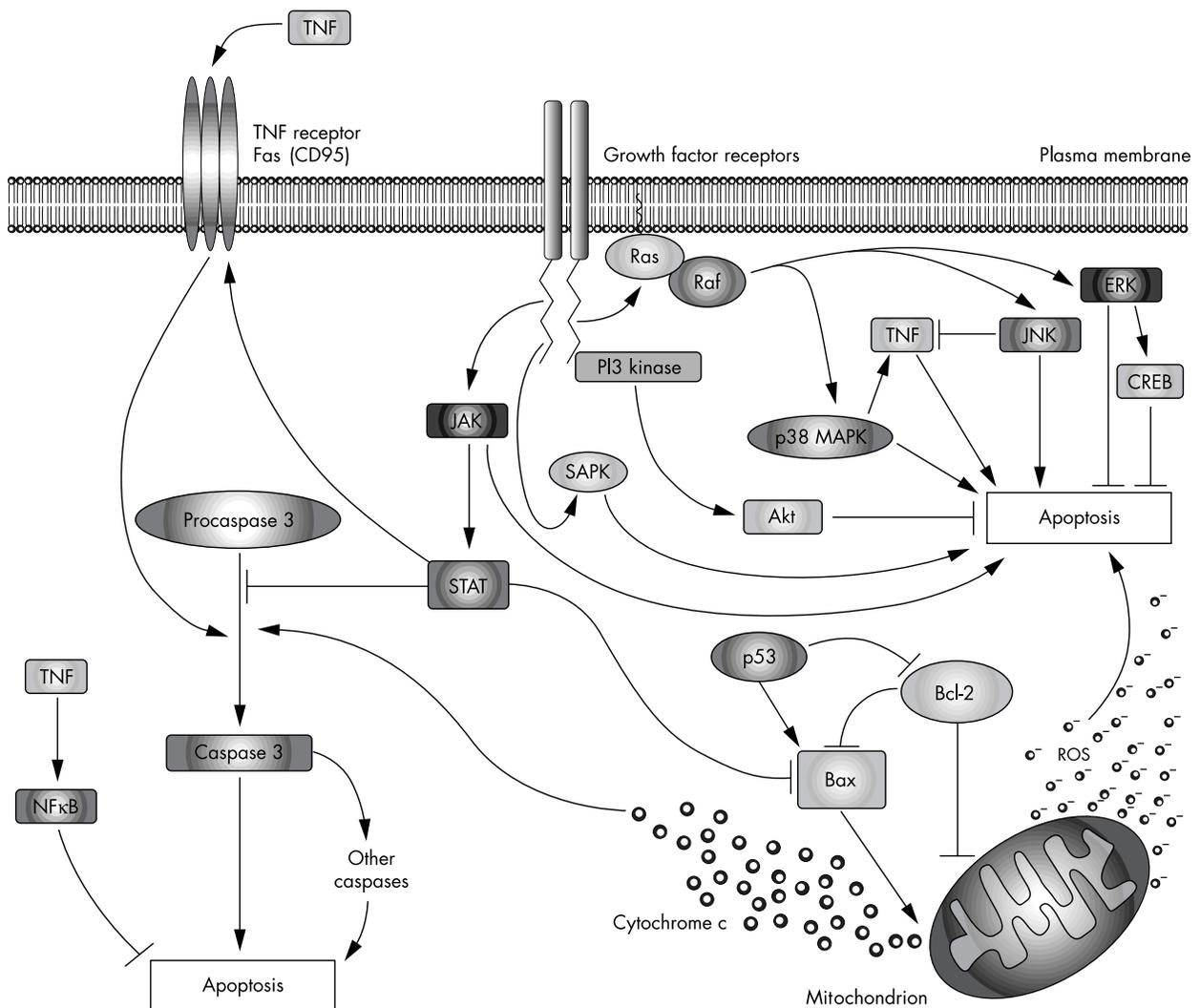
Figure 1 Overview of the signal transduction pathways related to ischaemia/reperfusion induced apoptosis in cardiomyocytes. In this figure, inducing or activating effects are depicted by an arrow and inhibitory effects are depicted by a blocked line. Akt, protein kinase B; ERK, extracellular signal related kinase; JAK, Janus kinase; JNK, Jun N-terminal kinase; MAPK, mitogen activated protein kinase; NFκB, nuclear factor κB; PI3 kinase, phosphatidylinositol 3'-kinase; ROS, reactive oxygen species; SAPK, stress activated protein kinase; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor.

activation of Mch2α (a caspase family member and the only known laminase[110]), which cleaves its substrate lamin A (a type of filament protein which, together with lamins B and C, forms the nuclear lamina of eukaryotic cells).[108]

p53

Exposure of neonatal rat cardiomyocytes to 24 hours of hypoxia resulted in an increase of apoptosis, as measured by DNA laddering, detection of DNA fragmentation by enzyme linked immunosorbent assay, and annexin V–FLUOS/ propidium iodide double staining.[111] Reoxygenation further increased the number of apoptotic cells. Hypoxia resulted in the upregulated expression of the bcl-2 and p53 proteins, whereas reoxygenation downregulated bcl-2 expression and further upregulated p53.[111] Chinonin, a natural antioxidant, significantly attenuated hypoxia/reoxygenation induced apoptosis, downregulated p53 expression, and induced bcl-2 upregulation.[111]

> "It has been suggested that p53 plays a crucial role in the induction of apoptosis as a result of hypoxia in cultured neonatal rat cardiomyocytes"

In another experiment in neonatal rat cardiomyocytes, 48 hours of hypoxia resulted in an increase of TUNEL positive cells and an increase in p53 protein, which was not accompanied by increased DNA laddering.[112] This was also accompanied by increased expression of p21/WAF-1/CIP-1, a well characterised target of p53 transactivation.[112] Because overexpression of wild-type p53 by adenovirus mediated gene transfer is sufficient to induce apoptosis in neonatal rat cardiomyocytes under normoxic conditions,[112] it has been suggested that p53 plays a crucial role in the induction of apoptosis as a result of hypoxia in cultured neonatal rat cardiomyocytes.

In contrast, equivalent apoptosis was measured in hypoxic/ acidotic cardiomyocytes isolated from the hearts of wild-type or homozygous p53 knockout mice.[36] However, in these mice severe chronic hypoxia alone was not sufficient to induce apoptosis, but had to be accompanied by a decrease in the extracellular pH to induce apoptosis, whereas reoxygenation resulted in increased apoptosis in a p53 independent manner. These results indicate that the fall in extracellular pH or reoxygenation, but not solely hypoxia, is responsible for the induction of apoptosis in murine cardiomyocytes, and that the process is a p53 independent one.

Table 2 Overview of proapoptotic and antiapoptotic mediators in ischaemia/reperfusion induced apoptosis in cardiomyocytes

Type of mediator	Human	In vivo animal	Isolated animal hearts	Isolated and cultured cardiomyocytes
Proapoptotic	Bax[37 39] Soluble Fas ligand[40]	Bax[15 41] Fas[17 43] ROS[60 61] p53[62] SAPK[17 66] JNK[67] p38 MAPK[67] TNF[79] Caspase 1[21 24 81–83] Caspase 3[21 24 81–83]	Fas[27] Bcl-2[92] ROS[29] p53[30] JAK[93] SAPK[94] p38 MAPK[31 97] JNK[97]	Soluble Fas ligand[27] ROS[35 108] p53[112] p38 MAPK[113 114] JNK[115] TNF-α[124] Caspases[32 103] Cytochrome c[32 103] Fas receptor[32] Vacular proton ATPase[34] Bad[109] Bax[109]
Antiapoptotic	Bcl-2[37–39] Soluble Fas[40]	Bcl-2[15 17 41] PKC[22 58 59] JAK/STAT[65] IGF-I[68 69] NO[70–72] HSP70[74] Calcineurin[75] TNF[80] Kallikrein[85]	ERK[97] αβ Crystalline[101]	TGF-β1[32] Mitochondrial ATP sensitive K+ ch.[109] MEKK1[114] JNK[114] HGF[116] ERK[117 119] PI3 kinase[118 119] Akt[118 119] HSP70[74 120–122] IGF-I[119] CREB[119] Bcl-2[32 119] Calcineurin[123] TNF-α[125]

Akt, protein kinase B; CREB, cAMP response element binding protein; ERK, extracellular signal related kinase; HGF, hepatocyte growth factor; HSP, heat shock protein; IGF, insulin-like growth factor; JAK/STAT, Janus kinase/signal transducer and activator of transcription; JNK, Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MEKK, MAPK kinase kinase; NO, nitric oxide; PKC, protein kinase C; ROS, reactive oxygen species; SAPK, stress activated protein kinase; TGF, transforming growth factor; TNF, tumour necrosis factor.

The role of kinases

In cultured neonatal rat cardiomyocytes, two distinct phases of p38 MAPK activation are seen during hypoxia; a transient activation that starts within 10 minutes of hypoxia and a sustained activation that starts after two hours of hypoxia.[113] In this model, ERK2 and JNK are not activated. By using the specific p38 MAPK inhibitor SB 203580, apoptosis was attenuated, even when only the sustained activation of p38 MAPK was inhibited. This suggests that the sustained activation of p38 MAPK induces apoptosis in neonatal rat cardiomyocytes after hypoxia.[113] In this study, however, the distinction between necrotic and apoptotic cells was made solely by differences in the morphology of their nuclei.

In agreement with this study, embryonic stem cell derived cardiomyocytes (ESCM) with targeted disruption of the MEKK1 (a MAPK kinase kinase) gene were extremely sensitive, compared with wild-type ESCM, to H_2O_2 induced apoptosis. p38 MAPK was suggested to play a proapoptotic role by activating the production of TNF-α,[114] whereas an antiapoptotic role was suggested for MEKK1. MEKK1 inhibits the production of TNF-α via the activation of JNK, suggesting an antiapoptotic role for JNK. In contrast, in rat neonatal cardiomyocytes transfected with antisense JNK DNA, condensation of the nuclei was inhibited after H_2O_2 treatment, suggesting a proapoptotic role for JNK.[115]

Treatment of both isolated adult rat cardiomyocytes and cultured mouse cardiomyocytes with hepatocyte growth factor (HGF), subsequent to oxidative stress, resulted in attenuated apoptosis, partially through the MEK–p44/42 MAPK pathway,[116] suggesting an antiapoptotic role for HGF.

In neonatal rat cardiomyocytes, it was suggested that ERKs have an antiapoptotic role in response to raised H_2O_2 concentrations after oxidative stress, through the activation of cyclooxygenase 2 expression and subsequent prostaglandin production.[117]

Insulin-like growth factor I

In rat neonatal cardiomyocytes, treatment with IGF-I increased the activity of phosphatidylinositol 3'-kinase (PI3 kinase) and Akt (protein kinase B).[118 119] Interestingly, in cardiomyocytes infected with an adenovirus carrying constitutively active forms of PI3 kinase or Akt, apoptosis is significantly inhibited after hypoxic treatment.[118] Indeed, in another study it was found that the inhibition of PI3 kinase or Akt abolished the antiapoptotic effects of IGF-I.[119] In neonatal rat cardiomyocytes, IGF-I did prevent apoptosis after hypoxia, which also could be suppressed with genistein, a tyrosine kinase inhibitor, and PD-098059, a MAPK inhibitor.[118] Subsequently, it was also found that IGF-I exerts its antiapoptotic effect through ERK1 and ERK2, resulting in activation of the transcription factor cAMP response element binding protein, which induces bcl-2 expression.[119]

Heat shock protein

A significant reduction of stress induced apoptosis was found in cardiomyocytes isolated from rat hearts overexpressing HSP70.[74 120–122]

Calcineurin

In H_2O_2 treated neonatal rat cardiomyocytes, apoptosis was almost completely abolished by endothelin 1, a growth promoting peptide.[123] In this study, apoptosis was measured by TUNEL, DNA laddering, morphological features, cytofluorometric analysis, and caspase 3 cleavage. Inhibition of calcineurin countered the antiapoptotic effects of endothelin 1, indicating a role for calcineurin in the antiapoptotic pathway of endothelin 1.

Tumour necrosis factor

In rat cardiomyocytes it was shown that physiologically relevant concentrations of TNF-α induced apoptosis (as measured by single cell microgel electrophoresis of nuclei and

by morphological and biochemical criteria).[124] The production of the second messenger sphingosine was also upregulated by TNF-α, indicating the possible involvement of sphingosine in TNF-α mediated cardiomyocyte apoptosis, and it was shown that treatment with sphingosine strongly induced apoptosis in rat cardiomyocytes.[124] However, in neonatal rat cardiomyocytes and H9c2 cells it was also shown that TNF-α activates both proapoptotic and antiapoptotic pathways.[125] This antiapoptotic signal was mediated via the translocation of NFκB.

DISCUSSION

In recent years much evidence has implicated apoptosis in myocardial cell loss in ischaemia/reperfusion injury. In vivo studies in humans and in animals, and in vitro studies in isolated animal hearts and cultured cardiomyocytes, all indicate that apoptosis contributes to the death of cardiomyocytes as a result of ischaemia/reperfusion injury. Here, we present an overview of recent studies into ischaemia/reperfusion induced apoptosis in cardiomyocytes in various models and of the molecular mechanisms underlying this process (fig 1; table 2).

One of the conflicting areas in the study of apoptosis in the heart is the mechanism of induction. In some studies, apoptosis was found to be induced by ischaemia alone, and after ischaemia followed by reperfusion,[6–8 10 12–15] whereas in other studies ischaemia alone was not sufficient to trigger apoptosis.[15 72 105] The absence of apoptosis during ischaemia can be explained by the notion that during ischaemia intracellular ATP stores are depleted. This absence of intracellular ATP might prevent the occurrence of apoptosis because apoptosis is an energy requiring process.

Studies are also conflicting with respect to the area of the heart in which apoptosis is found. In humans, apoptosis seems to occur primarily in the border zone of the ischaemic region[6–8 10] and, according to some studies, in the remote from ischaemia regions.[8 10] However, in vivo animal studies have demonstrated apoptosis both in the ischaemic region and the ischaemic border zone. In contrast, in some studies ischaemia caused apoptosis in the ischaemic region alone, whereas reperfusion caused a decrease in apoptotic cells in the ischaemic region and an increase in apoptotic cells in the ischaemic border zone and the remote from ischaemia regions.[13 14] These differences, with respect to the mechanism of induction of apoptosis and for the area in which apoptosis was found, theoretically could be explained by the different methods of measuring apoptosis that were used.

> "One of the conflicting areas in the study of apoptosis in the heart is the mechanism of induction"

The TUNEL staining method is now the most widely used marker for apoptosis in cardiomyocytes. However, it has become increasingly clear that the TUNEL assay is prone to false positive and negative results.[126] For instance, the staining kinetics of TUNEL is dependent on the reagent concentration, the extent of proteolysis, and fixation of the tissue. Furthermore, non-specific staining may be caused by active RNA synthesis and DNA damage in necrotic cells.[126] It is therefore important to standardise the TUNEL assay carefully by using DNAse treated tissue sections. Furthermore, these TUNEL positive cells must be characterised and a sufficient number of microscopic fields must be analysed. Even then, other methods of measuring apoptosis such as DNA laddering on agarose gels or the evaluation of apoptotic morphology should be combined with TUNEL to confirm the results.

The TUNEL method can also be used in electron microscopy analysis. In this method, the fragmented DNA is labelled using components of the ApopTag kit and subsequently with gold. In this way, nuclei can be analysed for TUNEL positivity and apoptotic morphology at an ultrastructural level at the same time.[105 127] However, a limitation of electron microscopic techniques is the small number of cells that can be analysed.

The detection of DNA fragments (DNA laddering) of sizes equivalent to mononucleosomes and oligonucleosomes is considered a trademark for apoptosis.[128–130] The analysis of these fragments on agarose gel results in a "ladder" pattern. In necrosis, DNA is cleaved at random, thus creating DNA fragments that vary greatly in size. On agarose gels, these fragments produce a smear. However, it is important to bear in mind the fact that in the heart cardiomyocytes are not the only cells that are prone to apoptosis. When DNA laddering is used to analyse cardiac homogenates, apoptotic granulocytes or endothelial cells may contribute to the laddering, making it uncertain that it results from the apoptosis of cardiomyocytes alone.[25]

Another often used marker for apoptosis is annexin V. Annexin V binds with high affinity to phosphatidylserine (after its translocation via the process of membrane flip flop) and labelled annexin V has been used to detect apoptosis both in vivo and in vitro.[11 26 105 131 132] It has been suggested that annexin V could have an immediate clinical use in patients for live image apoptosis monitoring.[11 131] An advantage of annexin V staining over the TUNEL method is that it can detect early apoptosis.[105] Indeed, cardiomyocytes stained with annexin V did not display late apoptotic morphology.[26] However, like TUNEL, annexin V might not be a totally specific marker for apoptosis because it can also bind to membrane fragments of late necrotic cells or flip flopped cells that have not undergone apoptosis/necrosis. Therefore, conclusions based solely on annexin V staining should be drawn with caution.

Thus, to date, there is no totally specific apoptotic marker that solely detects apoptotic cells, so that a combination of techniques should always be used to detect apoptosis.

Regardless of the limitations of the different methods for determining apoptosis, it is suggested that cardiomyocyte apoptosis as a result of ischaemia/reperfusion does not share all the classic hallmarks of apoptosis originally described for other cells.[3] For instance, electron microscopic analysis and histological studies have revealed only sporadic cardiomyocytes with classic late apoptotic morphology, such as the presence of apoptotic bodies.[25] This could be explained by the involvement of common mediators in both apoptosis and necrosis,[82] or an interchange between apoptosis and necrosis. It has been suggested that inflammatory cell activation could alter the process of apoptosis. Entman et al showed in reperfused canine myocardium that the presence of adhesion molecules on myocytes may allow the attachment of neutrophils and superoxide production, thereby converting apoptosis to necrosis.[133–136]

In line with this, in a recent study we have shown that cardiomyocytes that underwent flip flop of the plasma membrane (and were therefore annexin V positive but caspase 3 negative) bind secretory type II phospholipase A2 and subsequently potentiate the binding of the acute phase protein, C reactive protein (our unpublished data, 2002).

Another cause of the conversion of apoptosis to necrosis might be the depletion of ATP. In an ischaemia/reperfusion study, Lieberthal et al showed that the severity and duration of ATP depletion determines the mechanism of death, at least in proximal tubular cells: cells with an intracellular ATP concentration below a certain threshold become necrotic, whereas an ATP value above that threshold induces apoptosis.[137] It is likely that ATP plays a similar role in cardiomyocytes because ischaemia results in ATP depletion. Indeed, neonatal rat cardiomyocytes subjected to metabolic inhibition and subsequent ATP depletion favour necrotic cell death, whereas metabolic inhibition under ATP replenishing conditions increased the proportion of apoptotic cells, as determined by nuclear morphology and DNA fragmentation.[138] These authors suggested a model in cardiomyocytes in which intracellular ATP concentrations dictate the means of cell death—via necrosis

Take home messages

- Numerous studies indicate that apoptosis is a mediator of cardiomyocyte cell death after myocardial infarction
- Because there is no totally specific marker that detects apoptotic cells, a combination of techniques should always be used to detect apoptosis
- A lack of completion of the apoptotic process seems to be related to low concentrations of ATP and/or a conversion of apoptosis to necrosis by a secondary inflammatory response

when ATP is depleted and via caspase activated apoptosis when ATP is present.

"There is no totally specific apoptotic marker that solely detects apoptotic cells, so that a combination of techniques should always be used to detect apoptosis"

In conclusion, numerous studies indicate that apoptosis can cause cardiomyocyte cell death after myocardial infarction, although the exact mechanisms of apoptosis within the heart are not known and the interpretation of studies is somewhat difficult because of the different methods used to determine apoptosis. Nevertheless, the process of apoptosis in the heart has a unique position within the field of apoptosis, analogous to other ischaemically challenged processes: a lack of completion of the apoptotic process seems to be related to a lack of ATP and/or a conversion of apoptosis to necrosis by a secondary inflammatory response.

ACKNOWLEDGEMENTS

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