

ションを行っていく必要がある（表3, 4）。

ADLの評価法には、英国版Barthel Index（表5）などのように、家の中で動き回ったり、戸外に出るといった基本的機能としての基本的ADL（BADL）を評価するものと、Lawtonなどによる、移動能力や自家用車や公共輸送機関を利用しての手段的機

能、すなわち手段的ADL（IADL）を評価するもの（表6）などがある。

術後のリハビリテーションは身体的なものと精神的なものに分けられる。しかし、この両者は切り離して行われるものではなく、両者がうまくいって初めて効果をもたらすのである。その効果を上げるた

表3 入院によるADL悪化に関する因子

ベッド上安静 鎮静薬または睡眠薬 抗コリン薬 尿道カテーテルの長期使用 身体的抑制器具の使用
--

R. A. Rosenthal et al : Principles and Practice of Geriatric Surgery, Springer, P.187, 2001より引用, 改変

表4 入院によるADL悪化の予測因子

精神状態の不安定さ 入院前の身体的機能 入院前の社会的役割 自宅から外出する頻度

R. A. Rosenthal et al : Principles and Practice of Geriatric Surgery, Springer, P.187, 2001より引用, 改変

表5 英国版Barthel Index

機能	スコア	内容
1 排便	0	失禁・おむつ
	1	ときどき失敗（1週間に1回程度）
	2	自立
2 排尿	0	失禁・おむつ, またはカテーテルが必要（カテーテルを自分で処理できない）
	1	ときどき失敗（24時間に1回以下）
	2	自立（1週間以上にわたり1度も失敗がない）
3 洗顔	0	洗顔, 整髪, 歯磨き, ひげ剃りに介助が必要
	1	自立（用具の準備はしてもらってよい）
	2	全介助
4 便器の使用	1	部分介助
	2	自立（下着を脱いだり下ろしたりできる, 自分で履ける, 下着を上げたり着たりできる）
	0	全介助（口の中まで運んでもらう, あるいはなかなか飲み込むことができない, 経管栄養）
5 食事	1	部分介助（おかずを細かく切ったり, バターを塗るなど, 食べやすいようにしてもらえば, 自分で食べることができる）
	2	自立（食事は用意してもらってよい）
	0	起居不能（座位バランスがとれない）
6 起居・移乗	1	全介助だが, 座位はとれる（1, 2人の介助を必要とする）
	2	部分介助（1人で簡単に介助ができる, または監視・指示が必要）
	3	自立（監視・指示なしに自分でベッドからいすに移れる。またはその逆も可能）
7 歩行	0	歩行不可能
	1	車いすにて自立, 曲がり角もうまく曲がれる
	2	1人の介助で歩行可（監視・指示または身体を支えてもらう）
8 更衣	3	独歩可（補助具を使用してもよい。監視・指示は不要）
	0	全介助
	1	介助必要だが, 半分以上は自分でできる（ボタン, チャック可）
9 階段	2	自立（ボタンかけ, チャック, ひもを結ぶことなども可能）
	0	不可能
	1	介助必要（監視・指示, 体を支えてもらう, 昇降装置を使用するなど）
10 入浴	2	昇降自立（歩行のため補助具を使用してもよい。監視・指示は不要）
	0	介助
	1	自立（監視なしに浴槽に入浴でき, 自分で体を洗える。監視・介助なしでシャワーが浴びられる）
		合計得点 /20

Wade, D. T. : Measurement in Neurological Rehabilitation. Oxford Univ. Press, Oxford, P.175 ~ 178, 1992より引用, 一部改編

表6 手段的ADL評価

機能	スコア	内容
電話の使用能力	1	自分で電話がかけられる
	1	よく知っている2~3カ所ならかけられる
	1	電話の対応はできるが自分ではかけられない
	0	電話の使用ができない
買い物	1	自分で買い物ができる
	0	少しの物なら買い物ができる
	0	買い物に行くのに助けがいる
	0	買い物ができない
食事の準備 (男性の場合は「もしできれば」で、参考扱いとする)	1	食事の計画、準備、支度が自分でできる
	0	材料があれば食事の支度ができる
	0	食事が作れても、適切に食事が食べられない
	0	全面的に食事の準備に介助が必要
家事 (男性の場合は「もしできれば」で、参考扱いとする)	1	少しの助けて、または自分で家事ができる
	1	皿洗い、寝床の準備など簡単な仕事ができる
	1	簡単な仕事はできるが、掃除はできない
	1	家事に助けがいる
	0	家事がまったくできない
洗濯 (男性の場合は「もしできれば」で、参考扱いとする)	1	自分で洗濯ができる
	1	少しの物なら自分で洗濯ができる
	0	自分では洗濯がまったくできない
交通手段	1	自分で旅行ができる
	1	バスかタクシーに乗れるがほかの交通機関には乗れない
	1	助けがあれば公共の交通機関に乗れる
	1	助けがあれば少し旅行や移動ができる
	0	まったく旅行や移動ができない
薬の管理	1	自分で正しく薬が飲める
	0	前もって用意してもらえば、薬が飲める
	0	自分では、薬を正しく飲めない
家計の管理能力	1	自分で家計が管理できる
	1	日用品などは買ったりできるが、旅行や大きな買い物ができない
	0	家計の管理ができない
合計点		

Lawton, MP : Assessment of older people : Self-maintaining and instrumental activities of daily living. Gerontologist, P.179 ~ 168, 1969.より引用, 一部改編

めには、術前の評価に基づいて術後の早期から始めていく必要があり、実施に当たっては、患者と家族、医師、看護師、理学療法士、作業療法士などのリハビリテーションに携わるすべての関係者が病気に対する十分な理解を持ち、計画性を持ってチームワークよく行うことが大切である⁸⁾。

4. まとめ

高齢者では、合併症の発症を予防することがより大切となる。手術患者の管理は術前からすでに始まっているのであり、術前も含めた「周術期」の管理およびケアが重要である。さらに、術後の生活の質についても満足できるものとするため、術前の評価とそれに従った計画的な術前・術後ケアおよびリハビリテーションを、チームワークよく行っていく必要がある。

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6. 名古屋大学医学部附属病院外科病棟 における DVT 予防の実際

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1) 予防ガイドラインの作成および実施方法

当院では、2002（平成14）年にDVT、肺血栓塞栓症（PTEまたはPE）を予防するためのガイドラインを医療安全管理室と血管外科医師を中心にして独自に作成した。これは、①簡便にハイリスク患者をスクリーニングすること、②適切な対策方法を誰でも容易に選択できることを特徴としている。主には、①DVT/PE要因票（表1）を用いて重症度を点数化することと、②その点数に基づきDVT/PE予防方法（表2）に準じ対策を選択することの2段階で実施できる。

具体的には、まずDVT/PE要因票に基づき、治療要因と身体要因の2つの側面を点数化する。この合計が3点以上の場合、向血栓性素因のスクリーニングを中心とした血液凝固検査を追加し異常がないかを確認する¹⁾。さらに追加検査で血液凝固系の機能に異常を認めた場合は、すでに深部静脈血栓が存在していないかをカラードプラで確認し、異常があった場合は血管外科医師にコンサルトして対応策を講じる（表1）。

予防方法は点数にしたがって段階的に設定されており、高得点つまりハイリスク患者には弾性ストッキングや間欠的空気圧迫装置（intermittent pneumatic compression：IPC）の使用および低分子ヘパリン（非分画ヘパリンも可）の投与を勧めている。リスクに応じて複数の対策方法が設定してあり、その患者に対して実行可能な方法を選択することができる（表2）。

IPCはハイリスク患者が多い外科病棟やICUに配置されており、使用することになった患者では、病棟から手術室に持ち込み、手術中から使用している。

2) 手術前後のDVT/PE予防の実際

手術を受けることが決定したら、担当医師は外来でDVT/PE要因票に基づきリスクの程度を確認する。当院の外科で行われている手術はほとんどが予定時間

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表1 DVT/PE要因票 2003年2月版

治療要因	
1. 60歳以上かつ予定時間1時間以上の全身麻酔手術	2点
2. 40歳以上かつ予定時間3時間以上の全身麻酔手術	2点
3. 60歳以上で気腹を用いる手術	1点
4. 術後に下肢の運動を制限する必要がある(経大腿動脈動脈造影・血管内治療を含む)	1点
5. 術後などに水分制限下の安静を必要とする	1点
治療要因計 点(A)	
身体要因	
1. 過去に、深部静脈血栓症・肺塞栓症を起こした事がある	5点
2. 血縁者に、深部静脈血栓症・肺塞栓症を起こした人がいる	3点
3. 過去に、急に膝から下が腫れて痛くなった事がある	4点
4. 血縁者に、急に膝から下が腫れて痛くなった事がある人がいる	3点
5. 凝固制御因子の異常を指摘されたことがある	5点
6. 3回以上連続して流産を繰り返したことがある	3点
7. 1日の大半をベッド上で過ごす	1点
8. あまり水分を取らない/取らないように指示されている	1点
9. 妊娠している/経口避妊薬内服中である	1点
10. 1年以上ステロイドを内服している	1点
身体要因計 点(B)	
A+B= 点	
A+Bが3点以上の場合には、血液凝固追加検査	
異常の場合	
ア. ATⅢ、Protein C、Protein S活性値の測定(註：肝機能や経口抗凝固薬の影響を受ける)	5点
イ. D-Dimer(高値、かつ動脈瘤・左房内血栓など動脈内血栓性疾患がない場合)	5点
ウ. 抗β ₂ GPI抗体	5点
エ. 抗CL抗体(PT正常範囲でかつAPTTが正常値以下の低下認められた場合)	5点
註：アからエの検査は、結果が出るまでに○日間かかります。	
血液凝固追加検査に異常を認めた時	
下肢静脈還流検査カラードップラによる形態学的検査または静脈還流機能検査を行う	
異常の場合、血管外科受診で個別対応します。	還流は正常の場合
最終合計点 点	
処置予定日 20__年__月__日	
記入医師名：	記入看護師名：

表2 DVT/PE予防方法 2003年2月版

手術患者		チェックリスト
5点以上	GEC+IPC（病院備品）を継続的に使用する	<input type="checkbox"/> GEC購入
	術式により可能ならばLMWH投与を勧める	<input type="checkbox"/> IPCの使用申込
	この場合、LMWH適応外使用のインフォームド・コンセントを得ておく	◎ LMWH承諾 ◎ LMWH非承諾
	同意が得られなければLMWHは使用しない	<input type="checkbox"/> LMWH在庫確認
	LMWHの投与、持続点滴或いは皮下注で、歩行開始後3日後まで続ける	
	IPCの使用期間は術後2日か、歩行開始まで続ける	
	GECは退院まで装着し、退院後1ヶ月の装着を勧める	
4点	GEC+IPC（病院備品）	<input type="checkbox"/> GEC購入
	IPCは術中から術後1日、術後2日目から歩行開始までは用手マッサージ等	<input type="checkbox"/> IPCの使用申込
	GECは退院まで装着し、退院後1ヶ月の装着を勧める	
2～3点	GECを歩行開始まで使用	<input type="checkbox"/> GEC購入
	以後は、臥床時下肢挙上	
0～1点	適切な輸液、早期離床	
経大腿動静脈処置患者		チェックリスト
4点以上	GEC+用手マッサージ/足関節運動	<input type="checkbox"/> GEC購入
	GECは退院まで装着し、退院後1ヶ月の装着を勧める	
2～3点	GECを歩行開始まで使用	<input type="checkbox"/> GEC購入
	以後は、臥床時下肢挙上	
0～1点	適切な輸液、早期離床	
GEC：弾性ストッキング（足関節部圧迫圧20mmHg）		購入先
IPC：間欠的空気圧迫装置（AV-impulse、フロートロンなど）		使用申込先
LMWH：低分子ヘパリン75IU/kg/day		申請先

3時間以上の全身麻酔手術であり、手術後は1日の大半をベッド上で過ごすことになり、3点以上となる。このため手術を受ける患者のほぼ全例が弾性ストッキングを使用している。

患者への説明は、まず医師から手術の説明を行うと同時に合併症としてDVTが発症する可能性があること、その予防対策として弾性ストッキングを着用する必要があることを説明する。その後、看護師から体位変換・ベッド上での下肢の運動・早期離床など、手術後にストッキング着用とともに行うDVTを予防する

方法を説明する。

予防に用いる場合のストッキングは、男性でも一人で着用することが簡便なハイソックスタイプを使用している。患者の下腿周囲径を測定して、適切なサイズを選択している。

手術当日は出棟前に弾性ストッキングを着用して手術室へ入室する。帰棟後は体位変換とともに、臥床中の下肢の運動を促している。できるだけ早期に離床を進め、初回の歩行はストッキングを着用したままで、看護師が介助して行う。

その後、さらに離床を進め、トイレ歩行が自由に行えるようになるまではストッキングを着用している。乳房や甲状腺の手術のように手術翌日に自由に歩行が可能となる場合は術後2日目まで、開腹手術の場合は術後3日目から10日目ぐらいまではストッキングを着用している。

ハイリスクでIPCの使用が必要な患者も同様にストッキングを着用している。ベッド上で過ごす期間は6時間おきに30分間の空気圧迫をストッキングの上から行っている。ガイドラインでは1カ月程度のストッキングの着用を勧めているため、できるだけ長期間着用するように指導している。

IPCは4点以上の場合だけでなく、肥満体型で主治医から使用を指示される場合や生体部分肝臓移植手術のドナーに使用している。

ガイドラインの運用当初はストッキングを自費購入していた。しかし、2004（平成16）年4月から肺血栓塞栓症予防管理料として会計請求できるようになり、病院側から医療材料としてストッキングを準備しているため、予防対策が必要な場合は全員がストッキングを着用できている。

おわりに

2003（平成15）年2月から7月までに集積した457例の評価票で、凝固制御因子欠乏症（プロテインC or S）が5例以上あり、4例ではDVTの既往がなく、さらに2例では65歳以上の高齢者であった²⁾。モンゴロイドにはFactor V Leidenの報告がないことはよく知られているが、凝固制御因子欠乏症の有病率は意外と多いのかもしれない。

当院で行っているDVT/PE予防法がどれだけ有効であるかは、このガイドラインの試行前後の発症率を比較しなければ不明である。残念なことに、病院全体

ではガイドライン試行前のデータを得ることは困難である。また、現在提示している治療要因・患者要因の点数づけの有効性に関しては、今後詳細な解析を行う必要がある。

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Regulation of mouse GADD34 gene transcription after DNA damaging agent methylmethane sulfonate

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Abstract

The GADD34 gene is transcriptionally induced by growth arrest and DNA damage. However, the mechanisms underlying the transcriptional regulation are still unclear. We analyzed the promoter of mouse GADD34 gene and the methylmethane sulfonate (MMS)-induced transcriptional regulation of this gene. By introducing genome mutants, which were linked to the luciferase reporter, into NIH3T3 cells, we defined a 100-bp fragment upstream of the transcriptional initiating site as the minimal promoter of the GADD34 gene. Subsequent study revealed that CRE-binding site located in this minimal promoter was critical for MMS-induced transcription of the GADD34 gene. In vitro binding experiments showed that phosphorylated c-Jun was contained in the CRE/DNA complex. Overexpression of the dominant negative form of c-Jun led to a decrease of MMS-responsive promoter activity. From these results, we conclude that the CRE site of the GADD34 promoter is indispensable to the MMS-responsive *cis*-element that c-Jun is the essential transcription factor for MMS-stimulated regulation of GADD34 gene expression and that the upstream signaling is dependent on JNK.

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Keywords: GADD34; Transcription; DNA damage; Stress response; c-Jun

1. Introduction

In mammalian cells, the response to genotoxic stress enhances DNA repair, transient growth arrest, and/or apoptosis. The 5 GADD genes, GADD7, GADD33, GADD34, GADD 45, and GADD153, were originally isolated after ultraviolet irradiation in Chinese hamster ovary (CHO) cells (Fornace et al., 1989). The GADD34 gene is the human/hamster homologue of the mouse MyD116 cDNA that was subsequently isolated as a primary response transcript expressed during myeloid differentiation of mouse M1 myeloid leukemia cells (Abdollahi et al., 1991). Moreover, GADD genes have a growth-inhibitory function and one or

a combination of these GADD genes suppress cell growth (Zhan et al., 1994). The GADD34 protein has a region of homology with the herpes simplex virus-1 (HSV-1) protein 134.5 (Chou and Roizman, 1994; He et al., 1996). The carboxyl-terminal domain of gamma 134.5 has the structural and functional attributes of a subunit of protein phosphatase 1 (PP1) specific for eukaryotic translation initiation factor 2 alpha (eIF2 α), which it has evolved to preclude shut-off of protein synthesis. Recently, other groups (Novoa et al., 2001, 2003) and we (Kojima et al., 2003) have reported one function of GADD34 under endoplasmic reticulum (ER) stress. ER stress induces the GADD34 gene expression. GADD34 binds to PP1 and this complex specifically dephosphorylates eIF2 α , which precludes the shut-off of new protein synthesis.

Because GADD34 was originally cloned after genotoxic stresses, GADD34 appears to possess another function that relates to genotoxic stresses. We have shown that transfection of GADD34 enhances the phosphorylation of p53 at

Abbreviations: GADD, growth arrest and DNA damage; MMS, methylmethane sulfonate.

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Ser15, and induces p21/WAF1 transcription via the p53 binding site (Yagi et al., 2003). Further, we have found that GADD34 is engaged in the pathway, which suppresses cell growth by genotoxic stress (unpublished data). The next step to determine how GADD34 is regulated by genotoxic stresses. Here, we cloned mouse GADD34 genome and determined DNA sequence of GADD34. We analyzed the GADD34 transcription induced by genotoxic stress, MMS. We defined the MMS-responsive *cis*-element and transcription factors that induce GADD34 promoter activity.

2. Materials and methods

2.1. Cell culture and reagents

NIH3T3 cells from the American type culture collection (ATCC, Rockville, MD) were maintained in a 37 °C humidified atmosphere containing 5% CO₂ with Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (GIBCO). Methylmethane sulfonate (MMS) was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Genome library

We subcloned the lambda FIX II genome library (Stratagene) using the mouse GADD34 probe designed the –250- to +40-bp range (+1 was the relative start site of the GADD34 promoter) and amplified it by polymerase chain reaction (PCR) using the mouse genome as a template. The PCR amplified fragment was subcloned into a TA cloning vector (Promega) and sequences were confirmed by dideoxy sequencing. Restriction fragment mapping and preliminary DNA sequencing of various subclones revealed the organization of the mouse GADD34 gene.

2.3. Plasmid constructs

GADD34 promoter-containing reporters were constructed as follows. pGL3b –4.0 kbp was constructed via insertion of a 4.0-kbp fragment of the mouse GADD34 promoter region, from –4.0 kbp to +100 bp relative to the TATA box, into a firefly luciferase reporter vector, pGL3-basic, at BglII sites. The GADD34 5'-deletion constructs were all based on the promoter-less luciferase reporter gene vector pGL3basic. A series of scanning mutants between GADD34 –100 and +49 bp were prepared by PCR amplification using the mouse genome as a template, employing different mutated 5'-oligonucleotide primers and a 3'-primer. The oligonucleotides used in these mutagenesis reactions were as follows: pGL3b-0.2 kbp, 5'-TACGTGAGATCGACGGCTCT-3' and 5'-CAGCGC-GCCCTATAGCGTAC-3'; pGL3b-55bp, 5'-CCTTTTCCCAGGGACTTCCG-3' and 5'-CAGCGCGCCCTATAGCGTAC-3'; pGL3b-CRE, 5'-CGA-GTCTCGACCTCTCCGGT-3' and 5'-CAGCGCGCCCTATAGCGTAC-3'; and pGL3-TATA, 5'-

CTCCGGTGACAACAGCACAGCC-3' and 5'-CAGCGCGCCCTATAGCGTAC-3'. The fragments were subcloned into TA cloning vectors (Promega) and mutant sequences were confirmed by dideoxy sequencing. pCRE-luc, pAPI-luc and pTAL-luc vectors were purchased from Clontech. The pTAL-luc contains the firefly luciferase (*luc*) gene fused to a TATA-like (TAL) promoter region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter. The pCRE-luc contains the firefly luciferase gene and has five copies of the CRE-binding sequence fused to the pTAL-luc vector. The pAPI-luc contains the firefly luciferase gene and has five copies of the API-binding sequence. The mammalian expression vectors employed in this study were driven by the cytomegalovirus (CMV) promoter. Dominant-negative c-Jun (TAM67) and dominant-negative ATF-2 (ATF2-dn) expression vectors were a kind gift from Dr. Xiao. The dominant-negative CREB (kcreb)expressions vector was purchased from Clontech.

2.4. Transient transfection and reporter gene assay

NIH3T3 cells were planted onto 24-well plates at a density of 20,000 cells/well. Cells were transfected with 100 ng/well of plasmids by the effectene transfection reagent (Clontech). MMS treatment was started 24 h after transfection, and several hours later cells were lysed and luciferase assays in the lysates were measured by luminometer. The transfection efficiency was estimated via cotransfection with thymidine kinase promoter-driven Renilla luciferase reporter vector (pRL-TK plasmid, dual luciferase assay system, Promega).

2.5. Electrophoretic mobility shift assays

Nuclear extracts from either proliferating or MMS-treated NIH3T3 cells were prepared as described (Hasegawa et al., 1999). A total of 5 µg of the nuclear extract was incubated with 5 fmol of ³²P end-labeled double-strand oligonucleotides with a sequence corresponding to the region from –31 to –14 bp from TATA box in the GADD34 promoter. Incubation was carried out in 1 volume of 10 µl at room temperature for 20 min. All binding reactions contained 10 mM Tris-HCl (pH 7.5), 4% glycerol, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 1 mM MgCl₂, and 0.5 mg/ml of poly (dI-dC). For competition, 1 pmol of unlabeled oligonucleotides were incubated in the reaction. In supershift experiments, 1 µl of antibody was added 10 min before the labeled probe. The electrophoretic mobility shift assay (EMSA) products were separated on a 4% polyacrylamide 0.5 × Tris-borate EDTA gel at room temperature at 150 V for 90 min. The gel was dried and subjected to autoradiography. The oligonucleotides used in these experiments were as follows: CRE-probe: 5'-TCCGGTGACGTCAGCACAGCCC-3' and mutated CRE-probe: 5'-TCCGGTGACAAACAGCACAGCCC-3'. The antibodies directed against c-Jun, ATF-2 and CREB and phosphorylated c-Jun, ATF-2 and CREB antibodies were

purchased from Cell Signaling Biotech: anti-c-Jun antibody (#9126), anti-phospho-c-Jun antibody (#9164), anti-ATF-2 antibody (#9222), anti-phospho-ATF-2 antibody (#9221), anti-CREB antibody (#9192), and anti-phospho-CREB antibody (#9191). Anti-mouse IgG antibody was purchased from Amersham.

2.6. Immunoblotting

Total cell lysates from either proliferating or MMS-treated NIH3T3 cells were prepared as described (Xiao et al., 1999). Cells were washed with ice-cold phosphate-buffered saline (PBS) twice and lysed with an ice-cold lysis buffer containing 10 mM HEPES (pH 7.4), 30 mM NaCl, 2% glycerol, 0.2% NP-40, 0.3 mM MgCl₂, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml leupeptine. Cytoplasmic extracts and nuclear extracts were prepared as previously described. Twenty-five micrograms of lysates was electrophoresed on 8%, 10%, or 12% SDS-polyacrylamide gels. Proteins were detected using a chemiluminescent ECL kit (Amersham) with one of the following antibodies: anti-c-Jun antibody (#9126), anti-phospho-c-Jun (Ser63) antibody (#9164), anti-phospho-ATF-2 (Thr71) antibody (#9221), anti-phospho-CREB (Ser133) antibody (#9191), anti-phospho-p38 antibody (Thr180/Tyr182) (#9211), anti-phospho-SEK1(Thr223) (#9151, Cell Signaling Technology) or anti-GADD34 antibody (C-19, Santa Cruz).

2.7. Statistics

Geometric means are expressed with S.E.M. Statistical significance of the differences among various groups was evaluated by two-way repeated-measures ANOVA. Differences were considered to be significant when $p < 0.01$.

3. Results

3.1. Organization of the mouse GADD34 gene

Using the mouse GADD34 probe, two positive clones, lambda FIX II 13 and 26, were identified. Restriction fragment mapping and DNA sequencing of various subclones revealed the organization of the mouse GADD34 gene (Fig. 1). The exon–intron organization of the mouse GADD34 gene was determined by direct DNA sequencing using oligodeoxynucleotide primers. A given exon–intron boundary was indicated when the sequence from genomic clones diverged from that of the cDNA. The entire mouse

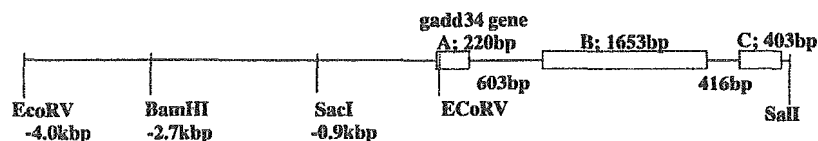


Fig. 1. Structure of the mouse GADD34 gene. Exon are indicated by boxes and designated as A, B and C. Restriction sites are shown.

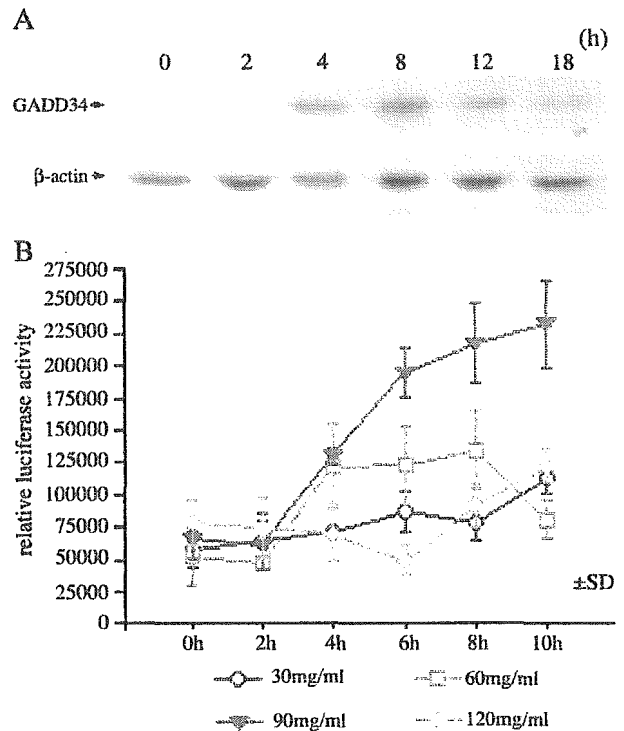


Fig. 2. Induction of GADD34 gene after MMS treatment. (A) Total cell lysates from either non-treated or MMS-treated NIH3T3 cells were obtained at appropriate time points (at 0, 2, 4, 8, 12 and 18 h). Proteins were detected by chemiluminescent ECL kit (Amersham). (B) Influence of MMS on the transcriptional activity of the mouse GADD34 promoter 4.0-kbp luciferase-reporter construct in NIH3T3 fibroblasts. NIH3T3 cells were transiently transfected with mouse GADD34 promoter 4.0 kbp (pGL3b-4.0 kbp) and stimulated with MMS (0, 30, 60, 90, 120 μg/ml) for various time periods. Results were correlated with renilla luciferase activity that came from co-transfected pRL-TK, and expressed as relative luciferase activity. The results were the mean of four transfections from two separate experiments. The error bars indicate the S.E.M.

GADD34 gene comprises three exons. The 900-bp-promoter region of the mouse GADD34 gene was completely sequenced from both strands. DNA sequences surrounding the mouse GADD34 gene transcriptional start site are highly GC-rich and have a TATA box.

3.2. Induction of mouse GADD34 promoter activity by MMS

MMS treatment invoked GADD34 expression at 4 h after MMS treatment. Activity peaked at 8 h and continued to 18 h (Fig. 2A). We investigated GADD34 promoter

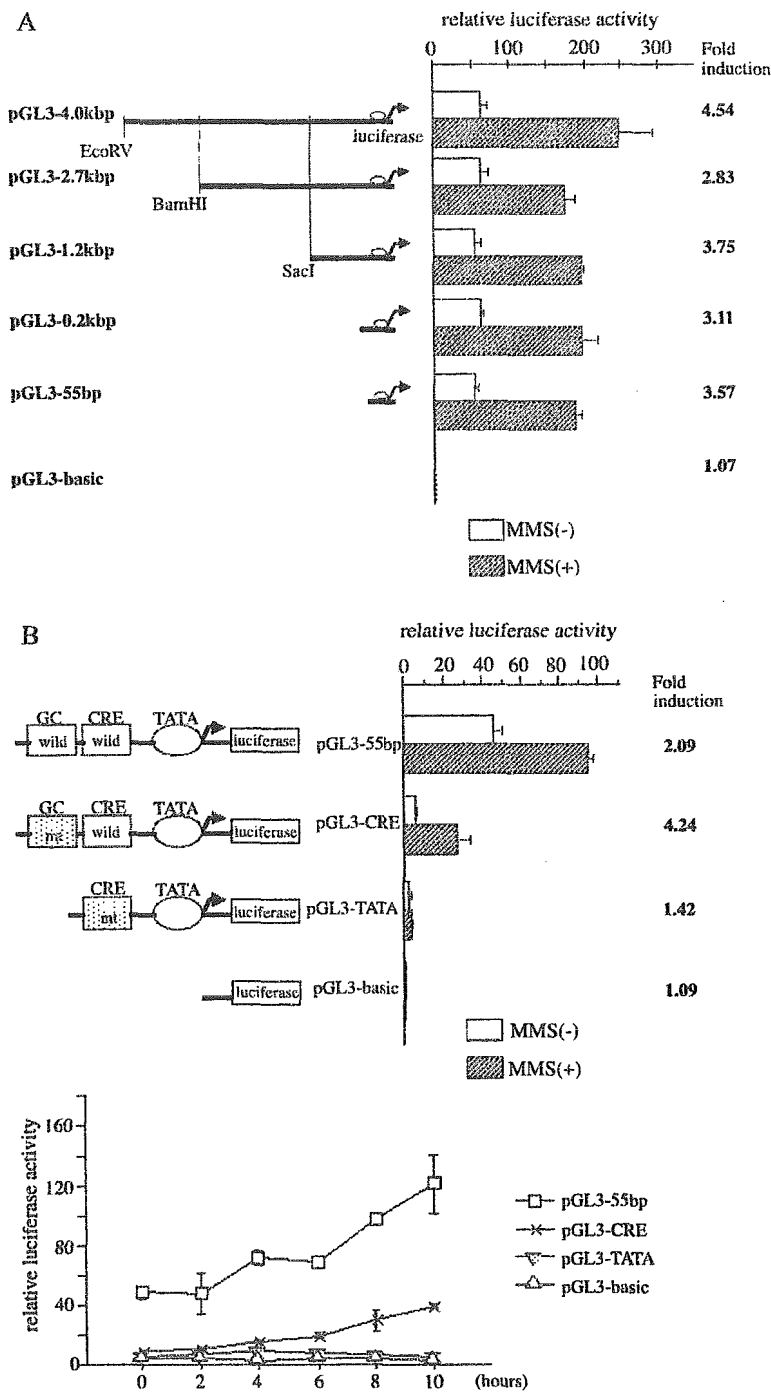


Fig. 3. Analysis of GADD34 5' -deletion mutants and mutagenesis of the mouse GADD34 core promoter in NIH3T3 cells. (A) NIH3T3 cells were transiently transfected with mouse GADD34 5' -deletion mutants of different length and incubated with or without MMS (90 µg/ml) for 8 h. Cell lysates were subsequently analyzed for luciferase activity. (B) The pGL3-55bp, pGL3-CRE, pGL3-TATA and pGL3-basic reporter constructs were transiently transfected into NIH3T3 cells. MMS stimulation and luciferase assay were performed as above. (C) Analysis of the transcriptional activity of the CRE consensus promoter vector (pCRE-luc) in MMS-treated NIH3T3 fibroblasts. NIH3T3 cells were transiently transfected with pCRE-luc, AP1-luc and pTAL-luc vectors and incubated with or without MMS (90 µg/ml) for 12 h. Cell lysates were subsequently analyzed for luciferase activity. The results were the mean of four transfections from two separate experiments. The error bars indicate the S.E.M.

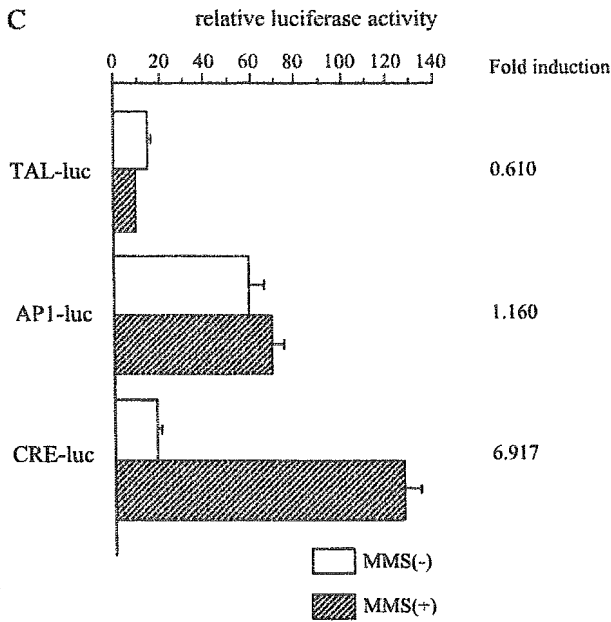


Fig. 3 (continued).

activity using the cells transiently transfected with pGL3b-4.0 kbp, driven by our longest mouse GADD34 promoter in the luciferase reporter gene. As shown in Fig. 2B, GADD34 promoter activity increased immediately and peaked at 10 h after incubation with a dose of 90 µg/ml MMS. Lysates from the cells transfected with pGL3b-4.0 kbp showed a 2.5-fold induction following treatment of 90 µg/ml MMS relative to the basal activity of this construct in untreated cells. A dose response was observed: increasing dose resulted in increasing promoter activity and the higher dose of MMS treatment delayed the maximal response. Because 120 µg/ml MMS resulted in 20% survival, we performed the following experiment with a dose of 90 µg/ml MMS.

3.3. Mapping of basal core promoter and MMS-responsive element in mouse GADD34 promoter

To elucidate the MMS-responsive regions of the mouse GADD34 promoter, NIH3T3 cells were transfected transiently with a series of 5' terminus-truncated mutants of the GADD34 promoter linked to the luciferase reporter gene.

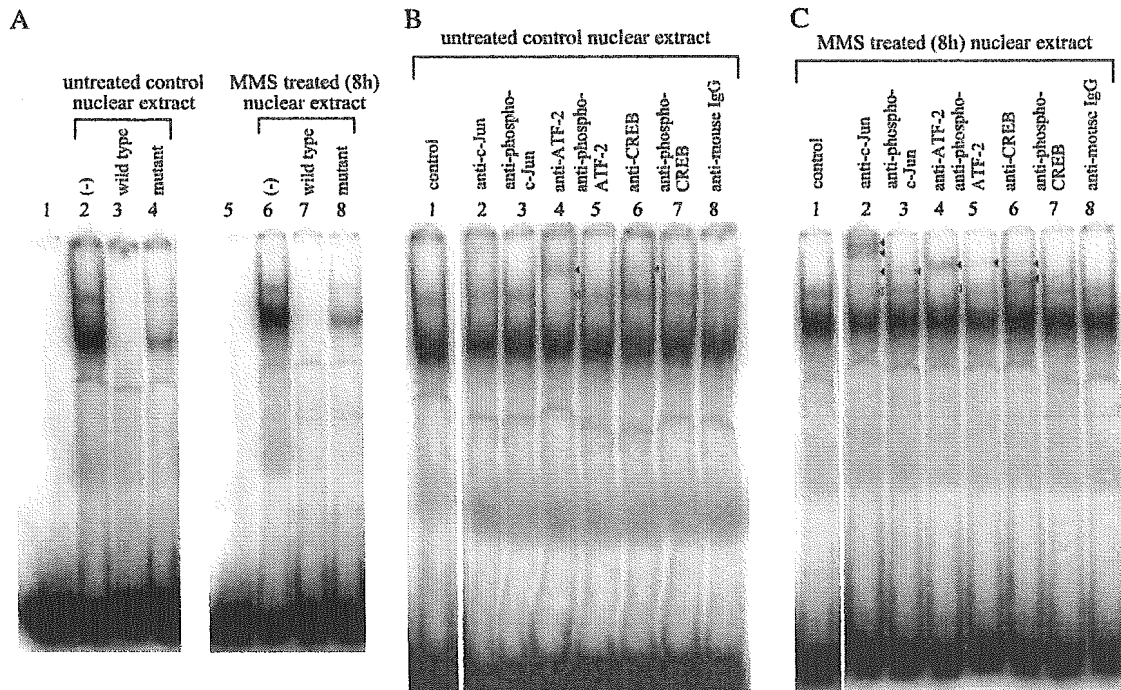


Fig. 4. EMSA of the mouse GADD34 - 31 to - 14 bp region. For EMSA experiments, double-strand oligonucleotides were synthesized and ³²P-end labeled. (A) Double-strand labeled probes were incubated with untreated NIH3T3 cell nuclear extracts (lanes 2 to 4) or MMS-treated nuclear extract (lanes 6 to 8), and in the presence of unlabeled (cold) consensus oligonucleotides (lanes 3 and 7) and mutant oligonucleotides (lanes 4 and 8). Lanes 1 and 5 were without nuclear extract. (B) For supershift assays, untreated NIH3T3 nuclear extracts were pre-incubated with antibodies prior to addition of the probe. Lane 1: without antibody, lane 2: with anti-c-Jun antibody, lane 3: with anti-phospho-c-Jun antibody, lane 4: with anti-ATF-2 antibody, lane 5: with anti-phospho-ATF-2 antibody, lane 6: with anti-CREB antibody, lane 7: with anti-phospho-CREB antibody, and lane 8: with anti-mouse IgG antibody. Solid triangle bar shows supershift complexes and open triangle bar shows diminished complexes. (C) MMS-treated NIH3T3 nuclear extracts were pre-incubated with antibodies prior addition of the probe. Lanes 1 to 8 numbering is same as (B).

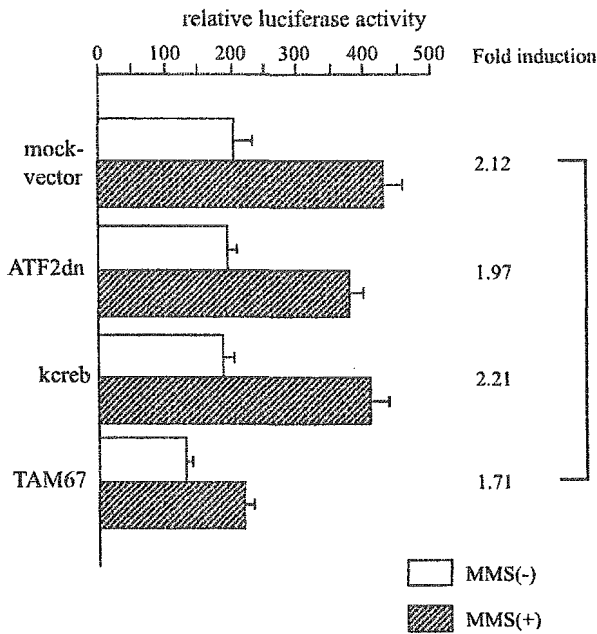


Fig. 5. Influence of dominant negative forms of c-Jun on GADD34 promoter activity in MMS-treated NIH3T3. Cells were transiently cotransfected with TAM67, ATF-2dn and kcreb expression vectors and pGL3-55 bp, and incubated with or without MMS (90 µg/ml) for 12 h. Cell lysates were subsequently analyzed for luciferase activity. The results were the mean of four transfections from two separate experiments. The error bars indicate the S.E.M. *Significantly different from control ($p < 0.01$).

As shown in Fig. 3A, the basal activities of the mutant promoters remained at the same level until truncation up to -55 bp (100-bp fragment, from -55 to -40 bp relative to TATA box; pGL3b-55 bp, 100 bp upstream of GADD34 initiation start site). The basal activity of constructs shorter than -55 bp dramatically decreased. 5'-Deletion analysis of GADD34 5'-flanking DNA revealed that -55 bp upstream of the GADD34 TATA box is able to confer full responsiveness to MMS. According to database analysis (TFSEARCH: Searching Transcription Factor Binding Sites (ver 1.3) Web site: <http://www.cbrc.jp/research/db/TFSEARCH.html>), we noticed that there are consensus protein binding elements in this region. A DNA-stretch spanning -42 to -33 bp is comprises putative binding sites for GC-box, and the region between -27 and -20 bp represented a consensus CRE/CREB element. A mutation in the GC-box site decreased basal transcription (Fig. 3B). These results suggest that the GC-box and the CRE located in the minimal promoter are the basal transcription element of the GADD34 promoter. On the other hand, a mutation in the CRE site decreased MMS-induced transcription. These results suggest that the CRE site located in the minimal promoter is the cis-responsive element for MMS-induced transcription of the GADD34 promoter.

To investigate whether the CRE site is the MMS-responsive element, either the pCRE-luc vector or pAPI-luc vector

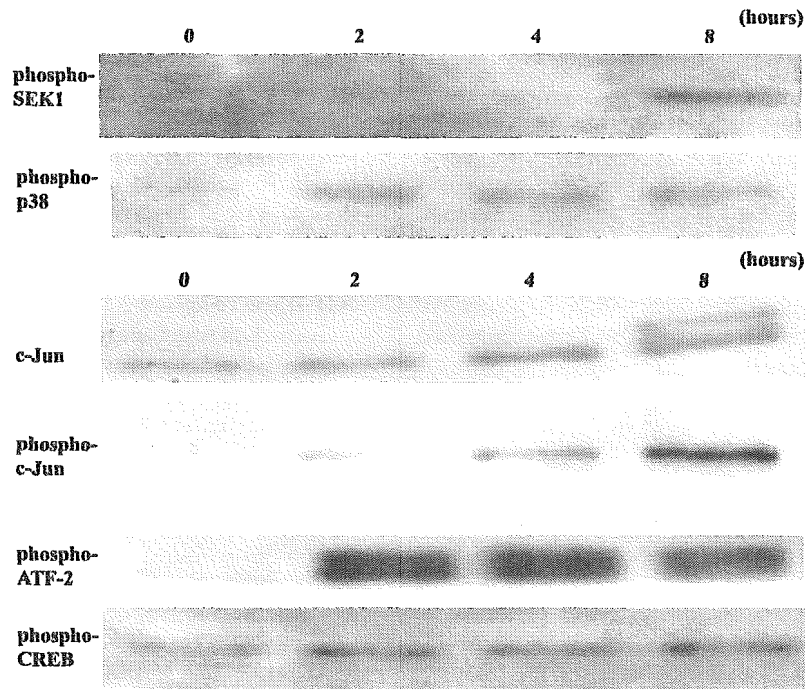


Fig. 6. MMS-treatment induced SEK1 activation and c-Jun phosphorylation. Total cell lysates from either non-treated or MMS-treated NIH3T3 cells were obtained at appropriate time points (at 0, 2, 4 and 8 h). Proteins were detected by chemiluminescent ECL kit (Amersham).

was transfected into NIH3T3 cells (Fig. 3C). Lysates from the cells transfected with pCRE-luc had a 6.9-fold induction of luciferase activity by the MMS treatment relative to the basal activity of this construct in untreated cells. Conversely, lysates from the cells transfected with pAP1-luc had only a 1.2-fold induction.

3.4. *c-Jun* binding to the CRE site increased by MMS treatment, while *ATF-2* nor *CREB* did not

As the sequence of CRE of GADD34 promoter was identical to the consensus sequence of CRE/CREB, we investigated by EMSA assay whether the complexes consisted of *c-Jun*, *ATF-2* or *CREB*. The addition of *c-Jun* (lane 2), *ATF-2* (lane 4) or *CREB* (lane 6) antibody to the binding reaction caused the complexes to be supershifted (Fig. 4A). By the MMS treatment, the amount of *c-Jun* or phospho-*c-Jun* supershift increased (Fig. 4B). Conversely, the amount of *ATF-2*, phospho-*ATF-2*, *CREB* or phospho-*CREB* supershift was not obviously changed by the MMS treatment.

3.5. Overexpression of dominant negative forms of *c-Jun* repressed GADD34 promoter activity by MMS treatment

In order to confirm the EMSA assay, we used the dominant negative form of *c-Jun*, *CREB* or *ATF-2*. When the dominant negative form of *c-Jun* (TAM67) expression constructs was co-transfected with pGL3b-55bp into NIH3T3 cells, reporter gene activity was repressed after MMS treatment (Fig. 5). However, neither dominant negative forms of *ATF-2* (*ATF2-dn*) nor *CREB* (*kcreb*) overexpression had any effect on MMS-induced transcriptional activity.

3.6. Upstream signaling of MMS-responsive GADD34 promoter activity

ATF-2 was phosphorylated at 2 h after MMS treatment, and continued to 8 h. Both protein amount of *c-Jun* and that of phospho-*c-Jun* (ser67) increased from 2 h and maximized at 8 h. *SEK1*, which phosphorylates *c-JUN* NH₂-terminal kinase 1/2 (*JNK1/2*) and activates *JNK*, was phosphorylated at 8 h after MMS treatment (Fig. 6).

4. Discussion

This is the first report to analyze mouse GADD34 promoter activity. Using 5' promoter deletion analysis, we have shown that the minimal basal activity of GADD34 is about – 55 bp from the TATA box. By site directed mutagenesis, we concluded that the CRE site is indispensable to the MMS-responsive *cis*-element. Both the GC-box and the CRE site are also found in tandem upstream of the TATA box of human and hamster GADD34 genes (Hollander et al., 1997). By EMSA analysis we have shown that *c-Jun*, *ATF-2* and *CREB*

specifically bound the GADD34 promoter element. Although three factors bind to the CRE site of the GADD34 promoter (Fig. 4A), only *c-Jun* and phospho-*c-Jun* increase the binding to the GADD34 CRE site at – 27 to – 20 bp by MMS treatment. The transcription factor *c-Jun* is one component of AP-1 that binds and activates transcription at AP1 sites. UV irradiation and growth factors stimulate phosphorylation of *c-Jun* and activate *c-Jun*-dependent transcription (Derijard et al., 1994; Kyriakis et al., 1994). As *c-Jun* gene expression depends on transcription factor *c-fos*, a specific inhibitor of MEK1 might decrease the expression of *c-fos*, but PD 98059 did not influence MMS-induced luciferase activity of the GADD34 promoter (data not shown). As consensus reporter vectors showed that the CRE site is more responsive than the AP-1 site, we concluded that CRE site is essential for MMS-stimulate regulation of the GADD34 promoter.

Here we analyzed transcription of the GADD34 gene by genotoxic stress. Recent findings show that GADD34 works under both ER stress (Novoa et al., 2001, 2003; Kojima et al., 2003) and DNA damaging stress (Yagi et al., 2003 and our unpublished data). Transcriptional regulation of GADD34 induced by both ER stresses and genotoxic stresses is important. *ATF3* has been recently shown to work as a transcription factor for the ER stress-induced GADD34 promoter by using the same CRE binding site (Jiang et al., 2004). ER stress inducer thapsigargin activates *PERK*, which phosphorylates *eIF2 α* . The phosphorylated *eIF2 α* stops new protein synthesis and induces GADD34 gene expression. GADD34 dephosphorylates *eIF2 α* and recovers from protein synthesis arrest. The question then becomes whether or not MMS treatment induces ER stresses. However, we found that MMS did not stimulate *eIF2 α* phosphorylation or induce the shut-off of new protein synthesis (unpublished data). GADD34 was transcriptionally expressed by the MMS treatment, which induces DNA damaging stress but not ER stress. Here, we concentrated on the transcription of GADD34 induced by genotoxic stress. GADD153, which is also induced by genotoxic stresses, uses the C/EBP-*ATF* composite site for transcription. The regulation of GADD153 gene transcription induced by arsenite was up-regulated by *ATF-4* and repressed by *ATF-3* (Fawcett et al., 1999).

Monofunctional alkylating agents like MMS are potent inducer of cellular stresses that lead to chromosomal aberrations and point mutations. Also such alkylating agents are ubiquitous in our environment, and are also produced endogenously by cells as natural metabolites. MMS has been shown to induce mitogen activation protein kinases (MAPKs) (Wilhelm et al., 1997). MAPKs comprise a ubiquitous family of tyrosine/threonine kinase and include extracellular signal-regulated kinase (ERKs), *c-JUN* NH₂-terminal kinase (JNKs) and p38MAPKs. Although some studies have revealed a relation between GADD45 or GADD153 and MAPKs, the relation between GADD34 and MAPKs has not yet been elucidated. Here we have shown that *JNK* is one of the upstream signals of GADD34 gene transcription.

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Protein phosphatase 1, but not protein phosphatase 2A, dephosphorylates DNA-damaging stress-induced phospho-serine 15 of p53

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Abstract Okadaic acid (OA) is a protein phosphatase (PP) inhibitor and induces hyperphosphorylation of p53. We investigated whether the inhibition of PP1 by OA promotes the phosphorylation of the serine 15 of p53. In vitro dephosphorylation assay showed that PP1 dephosphorylated ultraviolet C (UVC)-induced phospho-ser15 of p53, and that OA treatment inhibited it. One of the PP1 regulators, growth arrest and DNA damage 34 (GADD34), disturbed PP1 binding with p53, interfered with the dephosphorylation of p53 and increased the amount of phospho-p53 after UVC-treatment. This report provides the first evidence that PP1, but not PP2A, dephosphorylates phospho-serine 15 of p53.

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Keywords: Okadaic acid; DNA-damaging stress; p53 Phosphorylation; Protein phosphatase 1; Protein phosphatase 2A; GADD34

1. Introduction

Tumor suppressor p53 is tightly regulated by phosphorylation. The phosphorylated p53 induces cell-growth arrest and/or apoptosis through p53 response gene transcription. DNA-damaging stress, such as γ -irradiation and ultraviolet (UV) irradiation, activates ATM/ATR kinases, which in turn phosphorylate the serine 15 residue of p53 protein [1]. The phosphorylated serine 15 promotes the interaction with p300 [2], and p300 acetylated p53 and histones, which enhances promoter activity of p53 response gene. It remains unclear whether phospho-serine 15 stabilizes p53 protein. On the other hand, the serine 20 residue of p53 is phosphorylated by CHK proteins [3–5], and is the important site of interaction to MDM2 protein. Phosphorylation of serine 20 dissociates from MDM2, which promotes p53 stability [6].

It has been shown that multiple protein phosphatases (PPs) can dephosphorylate p53 in vitro. These phosphatases include

PP1, PP2A, PP5, Wip1 and Cdc14 [7–10]. Under normal condition, PP1/PP2A dephosphorylates the C-terminal of p53 protein; this site is phosphorylated by protein kinase C, which influences the DNA binding ability of p53 and transcriptional activity [11]. Meanwhile, the phosphorylation at the N-terminal of p53 is important for transcription and stabilization [12,13], but dephosphorylation of that site has not been well studied. Okadaic acid (OA), the inhibitor of PP1 and PP2A [14], accumulates hyperphosphorylated p53 [15,16] and the PP2A inhibitor, SV40 small t antigen, promotes the DNA binding ability of p53 and the transcriptional activity [17]. One of the PP1 regulators, growth arrest and DNA damage 34 (GADD34) (PPP1R15A, myd116), increases the amount of total and phosphorylated p53, and promotes p21 (waf1) mRNA expression [18]. These studies show that PPs regulate p53 through these phosphatase activities. Under stressed condition, however, the relations between PPs and p53 have been unclear.

In the present study, we show the relation between p53 and PP1 under DNA damaging conditions. We demonstrate that OA increases the amount of phosphorylation at the serine 15, which is related to the inactivation of PP1. Furthermore, by using GADD34 deficient MEFs, we show that the regulation of PP1 influenced p53 phosphorylation in vivo. These results suggest that PP1 is the key regulator of p53 and is the major phosphatase of phospho-serine 15 of p53.

2. Materials and methods

2.1. Cell lines and transfection

Human WI38 cells and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). Human p53 null cell lines SOAS-2 were maintained in modified McCoy's 5A medium (GIBCO) with 15% fetal bovine serum (FBS). All cells were grown at 37 °C in a humidified atmosphere of 5% CO₂. Transfections were performed with the effector reagent (Qiagen). Methyl methanesulfonate (MMS) was purchased from Sigma–Aldrich.

2.2. Preparation of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) from GADD34-deficient mice and wild-type mice were prepared from 14.5-day-old embryo [19]. All cultures were maintained in Dulbecco's modified essential medium (Sigma) with 10% FBS. Cells were plated 2×10^6 cells/10-cm plates for subculture.

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Abbreviations: OA, okadaic acid; GADD, growth arrest and DNA damage; UVC, ultraviolet C; MMS, methyl methanesulfonate

2.3. Plasmids

Human p53 expression vector was provided by Bai and Merchant [20]. PP1 cDNA was purchased from Invitrogen and cloned into the *Bam*HI/*Xba*I site of pcDNA3.1HisA vector with Xpress tag. Mouse GADD34 myc-tagged expression vector was described previously [18]. SV40 small t antigen cDNA was obtained by polymerase chain reaction (PCR) amplification using the cDNA of COS-1 cells as a template and employing 5'- and 3'-oligonucleotide primers. The oligonucleotides used in this experiment were as follows: small t antigen 5'-probe: 5'-CGGGATCCATGGATAAAGTTTAAACAGAG-3'; small t antigen 3'-probe: 5'-CCGCTCGAGTTAGAGCTTTAAATCTCTGTA-3'. The fragments were subcloned into the *Bam*HI/*Xba*I site of pcDNA3.1HisA vectors. Sequences were confirmed by dideoxy sequencing. When cDNA expression vectors were transfected, protein synthesis was inhibited by the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2 α). We employed pAdVantage vector (Promega) to preclude the termination of protein synthesis.

2.4. DNA-damaging and phosphatase-inhibitor treatments

WI38 cells and MEFs were grown to 50–80% confluence in 6-cm dishes in DMEM containing 10% FBS. DNA damage was achieved by exposing the cells to ultraviolet C (UVC) (50 J/m²) or MMS (80 μ g/ml). Cells were harvested at indicated time points. In some experiments, they were treated with increasing concentrations of OA (from 1 to 100 nM) 6 h before DNA-damage treatment. Four hours after irradiation, cells were treated with 10 μ g/ml of cyclohexamide to arrest new p53 protein synthesis and cells were then harvested at the indicated time points. OA and cyclohexamide were purchased from Sigma.

2.5. Immunoblotting

Proteins were analyzed by SDS-PAGE and detected by a chemiluminescent ECL kit (Amersham) with one of the following antibodies: anti-p53 antibody, anti-phospho-ser15 p53 antibody (Cell signaling), anti-GFP antibody (BD Biosciences), anti-GADD34 antibody (Santa Cruz), anti-eIF2 α antibody (Santa Cruz), anti-phospho-ser 51 eIF2 α antibody (BIOSOURCE international), anti-p21 (waf1) antibody (Santa Cruz), and anti- β -actin antibody (Amersham).

2.6. In vitro dephosphorylation assay

WI38 cells were UVC-treated (100 J/m²) and 1 h later the cells were lysed in low-stringency buffer (50 mM Tris-HCl, pH 7.5; 120 mM NaCl; 0.5 mM EDTA; and 0.5% NP-40) in the presence of PMSF and protease inhibitors. After pre-cleaning with protein G beads (Amersham), the extracts were immunoprecipitated with anti-p53 antibody in the presence of protein G beads for 4 h at 4 °C. The beads were then washed three times with low-stringency buffer, and twice with PP1 reaction buffer containing 1 mM MnCl₂ and 5 mM caffeine. One unit of PP1 (New England Biolabs, Inc.) was added to the immune complexes for 1 h at 30 °C. For inhibition studies, the immune complexes and PP1 were incubated with 1 μ M of OA in PP1 reaction buffer for 1 h at 30 °C. For positive control studies, Calf intestine alkaline phosphatase (CIAP) (Takara) was added to the immune complexes in CIAP buffer for 1 h at 37 °C. Dephosphorylation was analyzed by SDS-PAGE and was detected by immunoblotting with anti phospho-serine 15 of p53 antibody.

3. Results

3.1. Okadaic acid increases UVC-induced phosphorylation at serine 15 residue of p53

OA is an inhibitor of PP1 and PP2A. To evaluate the role of PP1/PP2A in regulating p53 phosphorylation after DNA-damaging stress, WI38 cells were exposed to UVC (50 J/m²) with increasing concentrations of OA. As shown in Fig. 1, the high concentrations of OA (100 nM) induced an increase in phosphorylated serine 15 of p53. On the other hand, the low concentrations of OA (1 nM) slightly reduced the phosphorylation of p53 after UVC treatment. The same treatment did not affect the amount of β -actin. These results demonstrate

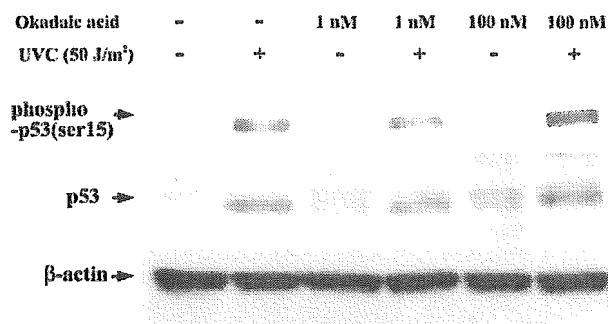


Fig. 1. OA increases p53 phosphorylation by UVC treatment. WI38 cells were treated by UVC (50 J/m²) for 4 h with increasing concentrations of OA. Phosphorylated p53 (top panel) and total p53 (middle panel) were determined. As a control, β -actin levels were determined (bottom panel). These are the representative data of more than two experiments.

that PP1 and/or PP2A regulated the phosphorylation at the serine 15 of p53 through phosphatase activity.

3.2. Protein phosphatase 1, but not protein phosphatase 2A, is the key regulator of phospho-serine 15 of p53

High concentrations of OA (100 nM) promoted the phosphorylation of p53, but it was unclear whether PP1 or PP2A was more important for the regulation of phospho-serine 15 of p53. To rule out the possibility that PP2A dephosphorylates p53, the PP2A inhibitor, SV40 small t antigen, was co-transfected to the culture cells to block p53 dephosphorylation. After UVC treatment, small t antigen did not affect the amount of p53, but induced a decrease in phospho-serine 15 of p53 (Fig. 2). To confirm the possibility that PP1 dephosphorylates p53, the PP1 regulator protein, GADD34, was co-transfected to the cultured cells to block p53 dephosphorylation. After UVC treatment, mouse GADD34 increased the amount of p53 and induced phosphorylation at the serine 15 of p53 (Fig. 2). These results suggested that GADD34 interfered in the dephosphorylation of p53, causing an increase in phospho-serine 15.

3.3. Protein phosphatase 1 dephosphorylates the serine 15 residue of p53 in vitro

Because we found that PP1 bound p53 in vivo, we next investigated whether PP1 could actually dephosphorylate phospho-ser15 of p53. We employed in vitro dephosphorylation assay. As shown in Fig. 3, PP1 dephosphorylated UVC-induced phospho-ser15 of p53. 1 μ M of OA treatment inhibited the phosphatase activity of PP1. CIAP also dephosphorylated p53.

3.4. The PP1 regulator GADD34 enhances DNA damage-induced p53 phosphorylation

To determine the relation between p53 phosphorylation and GADD34, we analyzed the p53 protein expression and p53 phosphorylation using GADD34 deficient MEFs. In wild-type MEFs, GADD34 was induced 8 h after MMS treatment (Fig. 4). And the protein levels of p53 and phospho-serine 18 of mouse p53 (corresponding to serine 15 of human p53) were highly expressed in a time-dependent manner at 8 h after MMS treatment. On the other hand, in GADD34-deficient MEFs, the levels of both p53 and phospho-serine 18 of p53

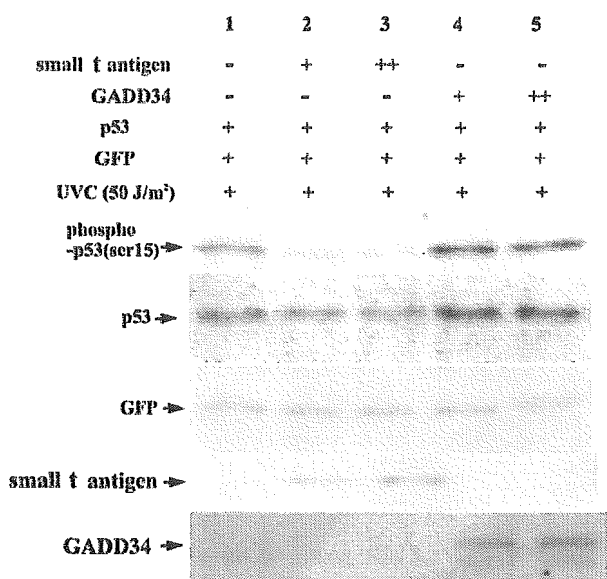


Fig. 2. Mouse GADD34 promotes phospho-ser 15 of p53 and interferes with the interaction between PP1 and p53. GADD34 promotes phosphorylation of p53 at serine 15, but SV40 small t antigen decreases phospho-serine 15. SOAS-2 cells were transfected with 0.1 µg of human p53 wild-type, 0.1 µg of GFP and 0.2 µg of Advantage vector (all lanes), and co-transfected with one or more of the following vectors: 0.2 µg of mock vector (lane 1), 0.1 µg of mock vector and 0.1 µg of small t antigen expression vector (lane 2), 0.2 µg of small t antigen expression vector (lane 3), 0.1 µg of mock vector and 0.1 µg of mouse GADD34 expression vector (lane 4), and 0.2 µg of GADD34 expression vector (lane 5). Phospho-ser 15 of p53 (top panel) and total p53 (middle panel) were determined. GFP was determined as the transfection internal control (bottom panel). These are the representative data of more than two experiments.

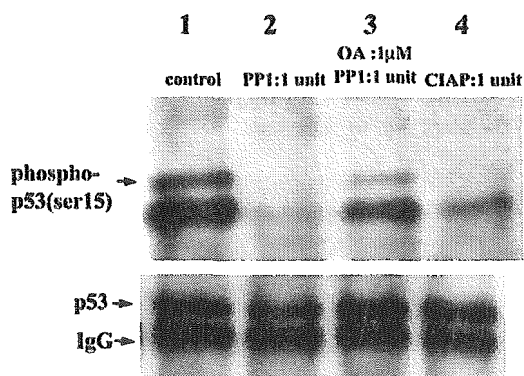


Fig. 3. PP1 dephosphorylates phospho-ser15 of p53 in vitro. WI38 cells were UVC-treated (100 J/m²) and p53 protein was immunoprecipitated with anti-p53 antibody. As a control, the immune complexes were incubated without PP1 (lane 1). One unit of PP1 was added to the complexes (lane 2). For inhibition studies, the complexes and PP1 were incubated with 1 µM of OA (lane 3). As a positive control, CIAP was added to the complexes (lane 4). The products were probed with anti phospho-serine 15 of p53 antibody (upper panel). Same membrane was re-probed with anti p53 antibody (lower panel). These are the representative data of more than two experiments.

expressions were lower than in wild-type MEFs. The fold induction of phosphorylated p53 per total p53 by MMS treatment was lower than that of wild-type MEFs. In GADD34-deficient MEFs, the level of p21 (waf1, cip1) expression was

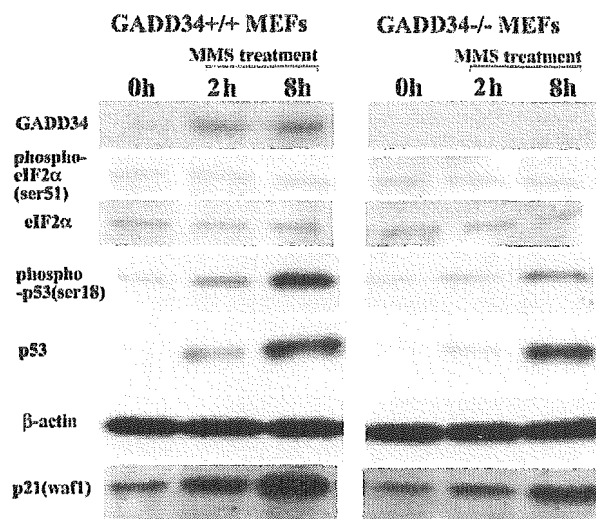


Fig. 4. Determination of p53 and phospho-p53 protein levels in wild-type and GADD34 deficient MEFs after MMS treatment. Cells were treated with 80 µg/ml MMS and cell lysates were prepared at the indicated time points. 50-µg lysate was run on 10% SDS-PAGE. To investigate the status of protein synthesis, we examined total eIF2α and phosphorylated eIF2α. Total p53, phosphorylated p53 and p21 (waf1) were determined by immunoblotting. As a control, β-actin levels were determined. These are the representative data of more than two experiments.

lower than that in wild-type MEFs. MMS treatment, unlike endoplasmic reticulum (ER) stress, did not induce the phosphorylation of eIF2α (Fig. 4). The same treatment did not affect the expression of β-actin, indicating that GADD34 protein did not have an overall positive effect on protein expression after MMS treatment.

4. Discussion

PP1 is the cellular protein serine/threonine phosphatase and the regulator of protein function through dephosphorylation. Here, we showed that OA treatment enhanced the phosphorylation of p53 at serine 15 after UVC treatment (Fig. 1). OA is the inhibitor of PP1 and PP2A, and this agent inhibits PP2A (50% inhibitory dose IC₅₀, 1–10 nM) more potently than PP1 (IC₅₀, 100 nM to 1 µM) [21]. Since 100 nM OA promoted the phosphorylation of p53, PP1 was thought to be more important in phospho-serine 15 of p53. To rule out the possibility that PP2A dephosphorylates p53, the PP2A inhibitor, SV40 small t antigen, was co-transfected with p53 expression vector to block p53 dephosphorylation. After UVC treatment, small t antigen did not affect the amount of p53, but induced a decrease in phospho-serine 15 of p53 (Fig. 2). The corresponding mechanisms remain unknown, but it is suggested that PP2A may act as a negative regulator of PP1 or activate the serine 15 kinase.

The PP1 regulator, GADD34 (PPP1R15A, myd116), is induced by ER stress [19,22,23], and GADD34 relocates PP1 to ER for dephosphorylation of eIF2α. We employed GADD34 to confirm the possibility that PP1 dephosphorylates p53. After UVC treatment, co-transfected mouse GADD34 expression increased the amounts of total and phospho-ser 15 of

p53 (Fig. 2). By using GADD34 deficient MEFs, we confirmed the relation between GADD34 and p53 phosphorylation (Fig. 4). In a previous study, we reported that PP1 regulator GADD34 induced phospho-serine 15 of p53 and promoted p21 (waf1) mRNA expression through p53 DNA binding sites [18].

The phosphorylation at the N-terminal site of p53 is important for transcription and stabilization [12,13], but the dephosphorylation of N-terminal site has not been well studied. Here, we revealed for the first time a relationship between N-terminal phospho-serine 15 residue of p53 and PP1, and that phospho-serine 15 temporarily promotes the stability of p53 protein. By *in vitro* dephosphorylation assay (Fig. 3), we showed that PP1 actually dephosphorylated phospho-serine 15 of p53. However, it is still unclear whether other phosphorylated sites of p53 are dephosphorylated by PP1.

In conclusion, we propose that in response to UVC treatment, p53 is phosphorylated by the serine 15 kinases and continuously dephosphorylated by PP1 but not by PP2A. This dephosphorylation requires the phosphatase ability of PP1. The PP1 regulator GADD34 interferes with the dephosphorylation of p53 and increases the phosphorylation at serine 15 of p53. These results support the speculation that GADD34 regulates p53 by modulating its phosphorylation levels.

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Toll-like receptor 4 is expressed with enteroviral replication in myocardium from patients with dilated cardiomyopathy

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Expressions of innate immune response proteins, most notably proinflammatory cytokines, against enteroviral (EV) infection have been documented in the heart of human dilated cardiomyopathy (DCM). Toll-like receptor 4 (TLR4) activates signaling pathways leading to the expression of proinflammatory cytokines implicated the etiology of DCM. We sought to determine whether EV replication activates TLR4-dependent immune response in myocardium obtained from patients with DCM. Endomyocardial biopsy tissues were obtained from 56 patients with DCM and 10 controls. Levels of plus- and minus-strand EV RNA and TLR4 mRNA were measured by real-time RT-PCR. Immunohistochemical analysis was performed to identify the cellular source of EV capsid protein VP1 and TLR4. Both plus- and minus-strand EV RNA were detected in 19 DCM patients (34%). Neither strand of EV RNA was detected in controls. TLR4 mRNA levels were higher in DCM patients than in controls ($P < 0.001$). A positive correlation was found between TLR4 levels and each strand type of EV RNA in EV RNA-positive patients (plus-strand vs TLR4: $r = 0.69$, $P < 0.001$; minus-strand vs TLR4: $r = 0.65$, $P = 0.002$). VP1/TLR4 double staining showed extensive colocalization of VP1 and TLR4 proteins in cytoplasm of cardiac myocytes in myocardium obtained from DCM patients. EV RNA-positive patients showed lower systolic function and larger ventricular volume compared with EV RNA-negative patients left ventricular ejection fraction (LVEF): $P = 0.002$; left ventricular end-systolic diameter (LVESD): $P = 0.004$. The DCM subgroup with high TLR4 levels showed lower LVEF and larger LVESD than the subgroup with TLR4 levels (both $P < 0.001$). This study suggests that myocardial expression of TLR4 associates with EV replication in human DCM. EV RNA and TLR4 mRNA levels may correlate with LV dysfunction in DCM. The expression of TLR4 against EV replication may be involved in the pathogenesis of DCM.

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Enteroviral (EV) RNA has been detected in a proportion of patients with dilated cardiomyopathy (DCM).^{1,2} It has been demonstrated that the detection of EV RNA in myocardium of DCM patients is associated with adverse prognosis.³ In particular, the presence of minus-strand EV RNA indicates active viral replication, and plays a role in the development of myocardial injury in DCM.⁴ Our previous study has reported that myocardial expression levels of tumor necrosis factor- α (TNF- α) and EV RNA were increased in patients with DCM regardless of etiology of left ventricular (LV) dysfunction.^{5,6} It is likely that an immune response against viral

pathogens is important in the pathogenesis of DCM. However, the precise mechanism underlying tissue injury in virus-mediated cardiomyopathy is not clear.

A family of toll-like receptors (TLRs) has recently been identified as a key component of pathogen-associated molecular pattern recognition machinery.⁷ At least nine types of human TLRs have recently been identified.⁷ It has been shown that active TLR4 led to expression of nuclear factor- κ B (NF- κ B)-controlled genes for proinflammatory cytokines that are required for activation of the immune response.⁸ TLR4 may therefore be an important factor in the signaling pathway in the host immune system in response to infectious disease. It has recently been reported that activation of antiviral immune response via TLR4 is important in the pathogenesis of viral infection.⁹

The purpose of this study was to determine whether TLR4 was expressed with EV replication,

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