表4　過去1年間の転倒を従属変数とした重回帰分析

	回帰係数	標準誤差	標準回帰係数	t値	p値
切片	.027	.101	.027	.269	.7881
つまづく	.225	.021	.246	10.740	<.0001
てすり必要	−.013	.022	−.014	−.589	.5560
歩行速度低下	.037	.022	.039	1.643	.1005
青信号中横断	.052	.030	.043	1.732	.0835
一km歩行	−.005	.025	−.005	−.205	.8377
片足立ち5秒	.021	.024	.023	.896	.3702
杖使用	.073	.024	.072	3.000	.0027
タオル絞れない	.103	.028	.086	3.656	.0003
目まい	.089	.021	.091	4.188	<.0001
猫背	−.001	.020	−.001	−.028	.9778
膝痛	.041	.020	.045	2.095	.0362
視力低下	.012	.020	.013	.606	.5444
難聴	.020	.019	.022	1.039	.2987
物忘れ自覚	−.024	.021	−.025	−1.150	.2503
転倒不安	.026	.023	.029	1.152	.2496
薬5種類	.004	.021	.004	.187	.8519
照明暗い	.022	.030	.015	.717	.4736
屋内障害物	.086	.023	.077	3.802	.0001
年齢	0.0004	.001	−.006	−.265	.7909
性	.019	.019	.020	.983	.3256

表5　ステップワイズ回帰分析

転倒対23独立変数　採用された変数

	回帰係数	標準誤差	標準回帰係数	除外F値
切片	.026	.016	.026	2.708
つまづく	.242	.020	.265	154.065
横断歩道	.059	.028	.049	4.345
杖	.085	.023	.084	13.576
タオル	.110	.028	.091	15.755
目まい	.098	.021	.101	22.596
膝	.053	.019	.059	7.811
障害物	.089	.023	.079	15.609

0.0001)，目まい(p<0.0001)，家の中に障害物がある(p=0.0001)，タオルがきつく絞れない(p=0.0003)，杖を使っている（p=0.0027)，膝が痛む（p=0.0362)，横断歩道の横断（p=0.08）抽出された（表4)．年齢，性を強制注入したステップサイズ回帰分析においても，同様の7因子が抽出された（表5)．

ロジスティック回帰分析による危険度(オッズ比)は，つまずく4.27倍，目まい1.77倍，タオルをきつく絞れない1.63倍，杖を使っている1.62倍，家の中に障害物がある1.57倍，膝が痛む1.4倍であった．横断歩道を青のうちに渡りきれないは1.27倍で有意には至らなかった（p=0.1)．

6) p≦0.1である7項目を暫定的に，転倒予測スコア短縮板として，得点による転倒率を計算すると，5項目以上該当する場合，60%以上に過去の転倒が見られた(図1)．

過去の転倒を判別する，カットオフ値を求めるため，縦軸に感度，横軸に1-特異度をとり，7項目の該当に関し，プロットした．カットオフ値2/3で感度65.1%，特異度72.4%が得られた（図2)．7項目中3項目の該当で，転倒危険者のうち2/3を検出し出来，特異度も70%以上で，スクリーニング検査としては満足出来る結果であった．

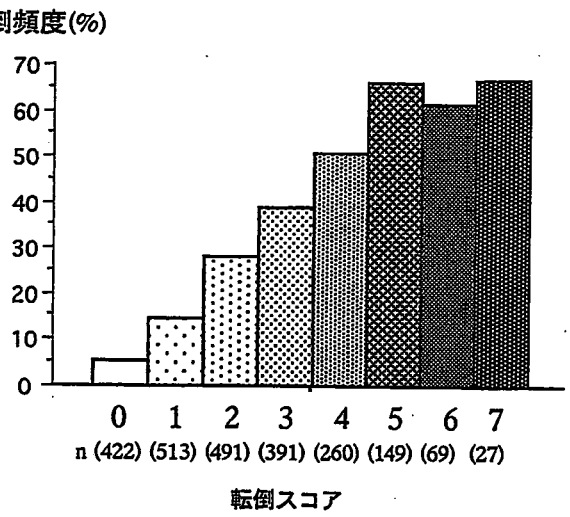

図1　転倒スコア得点別の転倒頻度
つまづく，目まい，タオルをきつく絞れない，杖を使っている，家の中に障害物がある，膝が痛む，横断歩道を青のうちに渡りきれないの7項目に該当する数（転倒スコア）を読横軸に表示．
縦軸は過去1年の転倒率を示す．

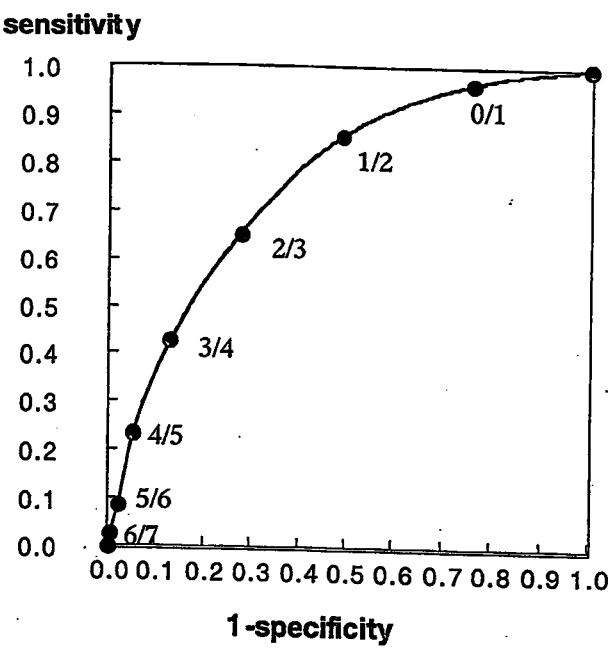

図2　7項目の「転倒スコア」のROC曲線
7項目の「転倒スコア」のカットオフ値を求めるため横軸に1-特異度，縦軸に感度をとり，該当項目数の1点相違ごとにプロットした（ROC曲線）．
カットオフ値2/3が最も優れている．

考　察

転倒は多数の内的要因，外的要因による，多危険因子の重層的な症候群（Multiple Risk Factor Syndrome）の一つである[1]．

ルベンスタインは，転倒に関する大規模研究のレビューを行い，筋力低下，バランス欠如，歩行障害，移動障害，ADL障害は殆どすべての研究で一致した危険因子であるが，視力障害，認知機能障害は半数の研究では危険因子として有意でなく，起立性低血圧は7研究中2つのみ有意であった[3]．このように，比較的人種や地域の差異が大きくないと予測される内的要因でも，危険因子としての重みには，対象によって異なる成績である．転倒の危険評価表の開発は，主として，介護施設[4]や病院[5)~7)]で行われ，過去の転倒，認知機能，感覚機能，運動・歩行機能，薬剤，立ちくらみ，慢性疾患が挙げられている．転倒の大部分は家庭内でおき，居間など室内で過半数がおきるとされているが，外的要因に関して，危険因子を標準化する試みは殆どない．地域における転倒危険因子の抽出は多く行われているが[9)~12)]，機能評価は質問紙表のみで完了せず，測定に人手を要するものが殆どである．また，内的要因と外的要因を公平に並べて，転倒の危険因子としての意味を調査した研究はなく，外的要因を加えた地域での簡易な危険因子評価表は見当たらない．

本研究では，過去の成績[1)~3)]及び，転倒評価表ワーキンググループの研究成績（未発表）から，内的要因の選択を行ったが，列挙は容易であった．外的要因に関しては，筋力低下，バランス欠如，歩行障害，移動障害，ADL障害と関連する外的因子に焦点を絞り，バリアフリーの観点から，障害物，段差，階段，坂道など多様な因子を下位項目に挙げた．視力障害と関連して，「部屋が暗く感ずるか」も加えた．

本研究において，段差，階段，坂道の項目は，転倒者と非転倒者の比較で差がなかった．意識されるバリアーは，転倒の危険因子でないことが示唆される．バリアーフリーは虚弱度が相当すすんだ対象のみに有効である可能性も否定できない．少なくとも，転倒といえばバリアフリーという喧伝された対応は，間違いであることが示された．

転倒者と非転倒者の比較では，段差，階段，坂道以外のすべての下位項目に有意差を認め，下位項目の選択基準が妥当であったこと（構成概念妥当性）が示唆された．転倒スコアの再現性も，比較的良好で，季節性を加味しても，リスクの相関が高いことが示された．

本研究では，過去の転倒を従属変数として，各因子が独立した危険因子であるかを検定した．

独立した有意な危険因子として，内的要因として，つまずく，目まい，タオルがきつく絞れない，杖を使っている，膝が痛むが抽出されたが，外的要因では，家の中に障害物があるのみであった．これらは，筋力低下，バランス欠如，歩行障害，移動障害，ADL障害[3]と関連する内的因子を具体的記述によって因子として捉えたものと評価されよう．最近の転倒の前向きコホート研究のメタアナリシスでは，信頼性のある方法で客観的に評価された下肢筋力低下（オッズ比1.76倍）と上肢筋力低下（オッズ比1.53）が抽出されている[13]．「つまずく」，「杖を使っている」，「膝が痛む」は下肢筋力低下の，「タオルがきつく絞れない」は上肢筋力低下の具体的表現といえよう．前述メタアナリシスではの複数回の転倒危険では，下肢筋力低下の危険度が（オッズ比3.06）上肢力低下（オッズ比1.41）より高かったが[13]，具体的記述である今回の下位項目でも，下肢筋力に関係ある項目が3項目のうち，「つまずく」4.27倍，上肢筋力の「タオルがきつく絞れない」1.63倍であったことと，一致した成績と考える．

外的要因では，「家の中に歩行上の障害物がある」という，比較的改善可能な因子であったことは，転倒予防に関連しても興味深い．今回，ロジスティック回帰分析にて，相対危険度（オッズ比）を求めたが，過去の成績では，転倒の既往は，転倒危険因子としてもっとも重要で，内外研究で3.8倍平均である[1]．

過去の転倒歴の頻度によって危険因子の相違を分析した成績では[14]，2回以上転倒者では，目まいがあると転倒率が2倍以上となり，重要な鑑別因子で，2回未満の対象では，ADL低下，上肢筋力低下，酒，痛み，活動度，教育などが転倒関連因子として抽出されている．転倒危険度の評価では，当然過去の転倒歴を因子に加え，転倒スコアを完成する必要がある．

結　語

内的因子及び外的因子を結合させ，設問によって解答可能な簡易な転倒リスク予測のための「転倒スコア」を開発し妥当性の検証を行った．今後より多くの，異なったフィールドで有効性が検証される必要がある．今回，下位項目の重回帰分析による絞り込みを試みた．「転倒スコア」の最終版の完成には，今回の結果では不十分であることは言うまでもない．本研究は縦断研究であり，1年後の観察期間終了後に，観察期間中の転倒を従属変数として再解析を行う予定である．

謝辞　本研究の転倒リスク予測のための「転倒スコア」は，転倒ハイリスク者の早期発見の評価方法作成ワーキンググループ（代表：鳥羽　研二）によって，開発された．メンバーに感謝したい．

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P: A classification tree for predicting recurrent falling in community-dwelling older persons. J Am Geriatr Soc 2003; 51 (10): 1356—1364.

Abstract

Development of a portable fall risk index for elderly people living in the community

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Aim: To develop a portable risk index for falls.

Methods: Risk factors were chosen from previously established factors then we added several environmental factors to the risk index; previous falls in the last 12 month, trippig or stumbling, inability to ascend or descend stairs without help, decreased walking speed, inability to cross a road within the green signal interval, inability to walk 1km without a break, inability to stand on one leg for 5 seconds (eyes open), using a cane, inability to wring out a towel, dizziness or faintness, stooped or rounded back, knee joint pain, visual disturbance, hearing disturbance, cognitive decline, fear of falling, receiving 5 or more prescribed drugs, sensation of darkness at home, obstacles inside, barrier on the carpet or floor, using steps daily at home, steep slopes around home.

Subjects: The questionnaire sheet was completed by 2,439 community-dwelling elderly subjects (76.3±7.4 years old). The frequency of each items of fall risk index was compared between fallers (history of fall within one year) and non-fallers. Multiple regression analysis was performed to identify independent risk factors for previous falls.

Results: Except barrier, step use and steep slope around home, all items in the fall risk index were more frequent in fallers.

Multivariate analysis revealed that tripping or stumbling, inability to cross a road within the green signal interval, dizziness or faintness, obstacles inside, inability to wring out a towel, cane use and knee joint pain were independent risk factors for previous falls.

These 7 selected items were further analyzed as predictors. The maximum sum of sensitivity and specificity was reached at the cut-off point of 2/3 (sensitivity 0.65, specificity 0.72) by receiver operating curve.

Conclusion: Portable fall risk index is useful for clinical settings to identify high-risk subjects.

Key words: *Falls, Community-dwelling people, Intrinsic factors, Environment, Fall index*
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〈原　著〉

痴呆高齢者に対する嚥下障害のスクリーニング方法の検討：簡易嚥下誘発試験と反復唾液嚥下テストの比較

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〈要　約〉　嚥下障害のスクリーニングのために，ベッドサイドで実施可能な簡便な検査法がいくつか提唱されてきている．しかしながら，痴呆を持つ高齢患者においては，それらの検査法の臨床的な有用性や限界について十分に検証されているとはいえない．今回，37例の入院患者（平均年齢81.8±1.2歳）を対象として，嚥下機能評価を，反復唾液嚥下テスト（repetitive saliva swallowing test，以下RSSTと略す）および簡易嚥下誘発試験（simple swallowing provocation test，以下SSPTと略す）を用いて実施し，同時に認知能と言語コミュニケーション能力について，改訂長谷川式簡易知能評価スケール（以下HDS-Rと略す）およびミニコミュニケーションテスト（以下MCTと略す）を用いて評価した．RSSTが実施できたのは22例のみであり(59%)，一方，SSPTは全例に実施可能であった．HDS-RスコアおよびMCTスコアは，RSST実施不可能群において，実施可能群に比べ有意に低値を示した（HDS-R：7±1 vs 15±3，$p<0.01$；MCT：47±8 vs 81±5，$p<0.01$）．また，RSSTにて異常反応は14例(64%)に，SSPTでの異常反応は5例(14%)に認められた．異常反応を示した患者では，認知能($p<0.05$)および言語コミュニケーション能力($p<0.05$)は有意に低下していた．また，SSPTにおいてむせのあるなしは，認知能の影響がみられた．この結果より，RSSTは高齢患者における嚥下障害の検出に有用であるが，その適応については患者の認知能と言語コミュニケーション能力に影響されることが示唆された．高齢者の嚥下障害についてその検査法を選択するうえで，老年医学的総合評価を行うことは有用であると考えられた．

Key words：高齢者，嚥下障害，嚥下スクリーニング検査，認知能，言語コミュニケーション能力

（日老医誌　2005；42：323—327）

緒　言

近年，高齢者人口の増加とともに高齢者の肺炎死亡例が増加している．その原因として不顕性誤嚥の関与が明らかにされてきており，高齢者における嚥下障害の適切な評価が重要な課題になっている[1)]．誤嚥の正確な評価については，嚥下造影が現状のゴールデンスタンダートであり，その後の治療方針の決定のために有用である[2)3)]．しかし，高齢者では，ほぼ全例が誤嚥リスクを有すると考えられ，これら全員に嚥下造影を行うことは，時間，労働力，医療費の諸点で無駄が大きい．そこで，肺炎の発症に結びつく誤嚥を生ずる可能性の大きい高齢者を絞り込む必要があるが，痴呆や身体機能の低下などの諸問題を有する高齢者においては，誤嚥リスク患者を識別する有用な嚥下障害のスクリーニング方法は確立してはいない[4)]．これまで，嚥下造影以外に，ベッドサイドでの嚥下や声の観察[2)5)6)]，オキシメータを使った嚥下評価[7)~9)]，咽頭反射の検出[9)~11)]，咳反射[12)13)]，反復唾液嚥下テスト（repetitive saliva swallowing test，以下RSSTと略す）[14)15)]，嚥下誘発試験[16)~18)]，水飲みテスト[19)~21)]，食物テスト[21)]，嚥下前・後X線撮影[21)22)]，内視鏡検査[23)]などの嚥下機能評価法が提案されている．スクリーニング検査は，通常，感度，特異度ともに優れていることが求められるが，痴呆を有する高齢者を対象とする場合，その検査法が，有効に実施できるか否かも重要である．そこで，高齢者を対象として，嚥下障害のスクリーニング方法として提案されておりベッドサイドで施行可能な，RSSTと，簡易嚥下誘発試験（simple swallowing provocation test，以下SSPTと略す）[16)17)]を行い，特に認知能，言語コミュニケーション能力との関連を検討したので報告する．

方　法

対象は，杏林大医学部附属病院高齢医学科に入院中の高齢患者で，嚥下障害が疑われ，検査に協力の得られた連続症例37例，男性18例，女性19例，平均年令81.8±

1) Y. Baba, H. Hasegawa, A. Machida, M. Akishita, K. Toba：杏林大学医学部高齢医学
2) S. Teramoto：東京大学医学部附属病院老年病科
受付日：2004. 7. 9，採用日：2004. 9. 14

1.2（標準偏差）歳であった．対象の入院時に診断されていた疾患の内訳は，脳血管障害60%，高血圧症57%，不整脈32%，虚血性心疾患19%，肺炎43%，糖尿病43%であった．各人が複数の疾患を持つ場合は，各々の疾患毎に数えた．対象のうち痴呆を示した例は31例であり，このうち痴呆の原因疾患として脳血管性痴呆20例，アルツハイマー型痴呆9例，分類不能2例であった．ADLについては，対象全例のバーセルインデックスの平均は29.8±35.4であった．

全例入院中に病状が安定した時期に，RSST，SSPTを施行した．施行時間帯は，午後の覚醒している時間とした．食事前後の2時間は避けるようにした．この2つの検査は同日に，その順序は無作為に行った．また，同時に認知能とコミュニケーション能力について評価した．RSSTは，既報のごとく，座位にて30秒間に唾液を何回嚥下できるかを評価し，2回以下を異常反応とした[14)15)]．SSPTは，既報のごとく，5Frの小児栄養チューブを鼻腔から約13cmまで挿入し，その先端が中咽頭にあることを確認した．チューブより0.4ml ないし2mlの5%ブドウ糖液を注入し，嚥下反射の誘発の有無と潜時を測定した．3秒以内に嚥下反射がみられた場合を正常反応とした[18)]．また，むせの有無も観察した．

認知能は，改訂長谷川式簡易知能評価スケール（HDS-R）[24)]を用い，言語コミュニケーション能力については，ミニコミュニケーションテスト（MCT）[25)]にて評価した．

統計計算について，群比較はANOVA検定後，有意差はStudent Neuman Keulsの検定を行いp値が0.05未満を有意とした．

成　績

RSSTについては，全例37例中，施行可能群（n＝22）と施行不可能群（n＝15）があった．この2群に対し，HDS-RとMCTを検討した．図1に示すように，HDS-Rでは，施行可能群vs施行不可能群：15±3点vs 7±1点（$p<0.01$）と，施行不可能群におけるHDS-Rスコアの有意な低下を認めた．MCTでは，施行可能群vs施行不可能群：81±5点vs 47±8点（$p<0.01$）と，MCTにおいても，施行不可能群におけるMCTスコアの有意な低下を認めた．以上から，認知能および言語コミュニケーション能力の低下により，RSST施行不可能例が増える事が確認された．

RSST施行可能群（n＝22）における正常反応群（n＝8，平均3.4±0.7回）と異常反応群（n＝14，平均1.3±0.8回）における認知能および言語コミュニケーション能力を検討したところ，図2に示すように，HDS-Rでは，

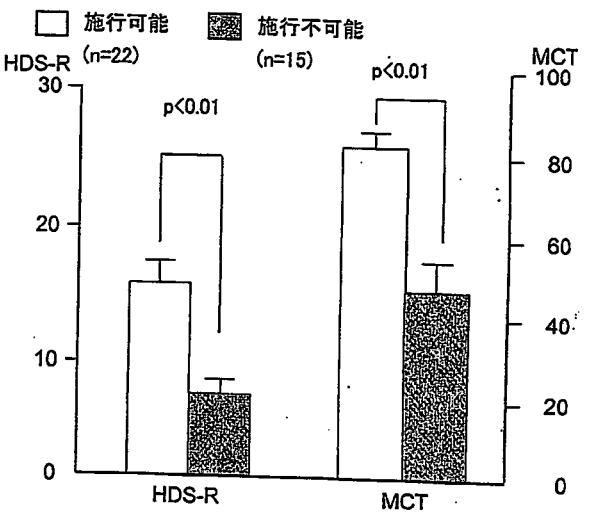

図1　反復唾液嚥下テスト（RSST）における施行可能群と不可能群の認知能および言語コミュニケーション能力の差異

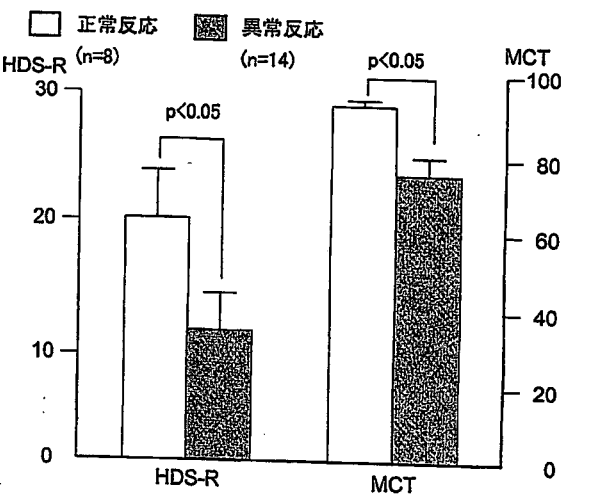

図2　反復唾液嚥下テスト（RSST）施行可能群における正常反応群と異常反応群の認知能および言語コミュニケーション能力の差異

正常反応群vs異常反応群：20±4点vs 11±3点（$p<0.05$）と有意に差を認め，異常反応群でHDS-Rスコアの有意な低下を認めた．MCTにおいても，正常反応群vs異常反応群：92±1点vs 76±6点（$p<0.05$）と有意な差を認め，異常反応群でMCTスコアの有意な低下を認めた．

一方，SSPTは，全例施行可能であった．RSSTにて正常であった8例は，SSPTではすべて正常反応を示した．RSSTにて異常反応を示した14例のうち，SSPTの正常反応を示したのは13例であり，SSPTにて異常反応を示したのは1例であった．また，RSST施行不可

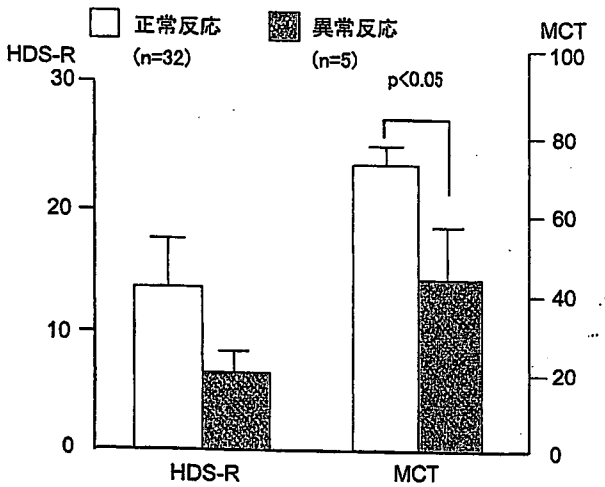

図3　簡易嚥下誘発試験（SSPT）における正常反応群と異常反応群の認知能および言語コミュニケーション能力の差異

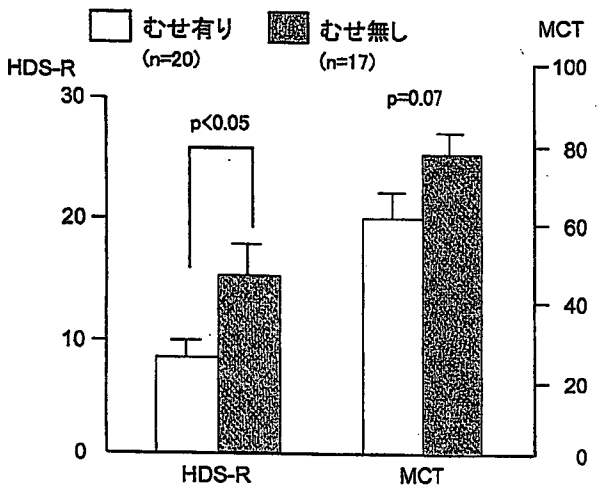

図4　簡易嚥下誘発試験（SSPT）におけるむせの有無と認知能および言語コミュニケーション能力の差異

能群15例のうち，SSPT正常反応例は11例，SSPT異常反応例は4例であった．

図3に示すように，SSPTにおいて，正常反応群（n＝32，平均1.5±0.7秒）と異常反応群（n＝5，平均10.0±8.3秒）における認知能および言語コミュニケーション能力を検討したところ，HDS-Rでは，異常反応群でHDS-Rスコアの低下を認めたが，正常反応群vs異常反応群：12±5点vs 7±2点（p＝0.189）と有意差は認めなかった．MCTにおいては，正常反応群vs異常反応群：73±5点vs 44±12点（p＜0.05）と有意な差を認め，異常反応群でMCTスコアの有意な低下を認めた．

SSPTにおける「むせ」のあるなしについては，正常反応群では「むせ」のあった例は47%，「むせ」のなかった例は53%であった．異常反応群では，「むせ」のあった例は80%，「むせ」のなかった例は20%であり，SSPT異常反応群にて有意に「むせ」の出現が多くみられた（p＜0.05）．

次にSSPTにおける「むせ」の有無と認知能，言語コミュニケーション能力との差異を検討したところ，図4に示すように，HDS-Rにおいては，「むせ」のある群vs「むせ」のない群：9±1点vs 15±2点（p＜0.05）と，「むせ」のない群でのHDS-Rの有意な上昇を認めた．MCTにおいては，「むせ」のない群でのMCTスコアの上昇を認めたが，「むせ」のある群vs「むせ」のない群：60±9点vs 78±4点（p＝0.07）と，有意差はみられなかった．

考　察

近年，高齢者肺炎における誤嚥の重要性が国内外で認知されてきた[1]．杏林大学医学部附属病院高齢医学科においても入院患者で肺炎は約17%にみられ，さらにそのうち誤嚥が原因となっているものが約42%であった（未発表データ）．誤嚥性肺炎は，症状が非定型的で，発熱，気道症状がないことがある一方，予後が不良である．したがって，誤嚥性肺炎を生じやすい嚥下障害を早期に検出し，嚥下リハビリテーションや誤嚥対策を行うことが，高齢者肺炎の予防・治療の点から重要と考えられる．しかし，多数例の高齢者に実施可能な有用な嚥下スクリーニング方法は確立されていない．

リハビリテーションの立場から重視されるベッドサイドでの嚥下，声の観察評価は，嚥下造影で検出できる異常を見逃す確率が高いことが知られ，また，評価する検査技師の能力によって異常の評価が異なる点も問題となる[2][6]．オキシメータによる嚥下障害の評価は，SaO_2の低下という客観的指標を用いるが，これ自体は嚥下障害そのものを反映するわけではなく，誤嚥や息止め，呼吸器疾患，心不全などの要素によって影響を受けるため，嚥下障害の評価法としては，極めて間接的である[7][8]．咽頭反射は最も簡便な評価法であるが，嚥下機能を直接反映するわけではないため，誤嚥評価には適さないことが報告されている[11]．これらの方法に比べ，近年提唱されたSSPT，RSSTなどの方法は，新たな嚥下機能障害評価法として注目されている．そこで，本研究では，これらの新しい嚥下機能障害評価法が痴呆のある高齢者についても応用可能か否かについて検討した．

その結果，SSPTは今回対象とした全例に安全に検査を施行できたのに対し，RSSTは一部の被験者には，検

査ができなかった．被験者の認知能と言語コミュニケーション能力は，SSPT施行については影響を与えなかったが，RSST施行については影響がみられた．SSPTが被験者の能動的な動作を必要としないのに対し，RSSTは被験者自身が指示を正しく理解し反復嚥下をする必要があるため，このような差が生じたものと考えられた．しかし，検査の施行にあたり，SSPTは，蒸留水（または，5%ブドウ糖液）や経鼻細管（小児用栄養チューブ）などの準備や器具を必要とするため，外来や在宅で実施する際の簡便さについては，RSSTに劣ると考えられた．

また，RSSTでの異常反応群や施行不可能群においてSSPTでは正常反応を示す例が多数みられ，検査法により嚥下機能の評価に差がみられた．これは，RSSTにて正常反応を示さないことのみでは誤嚥性肺炎の危険について判断できないことを示唆している．嚥下の時相のどこに障害があるかといった嚥下障害の質的な違いのみならず，痴呆の有無などを考慮した上で，多角的に嚥下評価を行う必要があることを示唆している．

このように高齢者においては認知能や言語コミュニケーション能力の状態に適した，より有用な検査法を選択することが必要と考えられた．今回は，RSSTとSSPTの二つの方法しか検討していないが，この他にも，様々な方法が提唱されているため，今後は，他の方法との検討を重ねて痴呆患者の嚥下障害の検出に有用な戦略を確立する必要があると考えられた．

本研究においてSSPTで評価された嚥下反射の潜時とHDS-Rの得点，言語コミュニケーション能力の評点とは関連がみられた．従って，高齢者の嚥下反射は認知能力や言語コミュニケーション能力によって大きな影響を受ける可能性が考えられた．これまで，痴呆と嚥下反射の関連については必ずしも明らかにされていない．脳梗塞発症後，その直後は嚥下反射が低下しているが脳梗塞の回復にともなって嚥下反射も改善することが報告されている[26]．この際，認知能も改善すると考えられるが，この両者の改善は，神経機能の改善が多面的に生じた結果と推察される．RSSTについても，唾液反復回数と認知能，言語コミュニケーション能力の評点とは関連がみられた．しかしながら，痴呆や言語コミュニケーション能力を評価しただけでは，嚥下障害をスクリーニングすることはできないため，RSSTやSSPTのような嚥下障害特異的なスクリーニングを必ず並行して実施する必要があると考えられる．

また，これまで，誤嚥の徴候としての「むせ」の有無が嚥下機能評価や摂食訓練の場で重要視されてきたが，今回の検討においても，SSPT異常反応群において，有意に「むせ」の発現がみられたことは，「むせ」が初期の嚥下障害の有効な判定材料となり得る可能性が示唆されたといえる．さらに，今回の研究では「むせ」の有無と認知能に有意な関係がみられた．「むせ」と咳反射は，嚥下機能評価において同様の意義があるといえるが，咳反射の減弱が誤嚥につながり，咳反射を強めることは誤嚥性肺炎の予防になるとの報告がある[11][12]．すなわち，「むせ」がないことは，嚥下機能が良好で誤嚥がない場合と，誤嚥があったとしても「むせ」のおこらない，より重篤な不顕性誤嚥の状態である場合とがある．「むせ」がある群の認知能が低下していたことは，認知能低下群で嚥下機能の低下が示唆されるが，より重症の痴呆では誤嚥しても「むせ」のない不顕性誤嚥の割合が増えるかどうかは今後の検討課題である．

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Abstract

Characteristics and limitation of portable bedside swallowing test in elderly with dementia：Comparison between the repetitive saliva swallowing test and the simple swallowing provocation test

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Several bedside portable swallowing tests have been advocated for screening for dysphagia. However, the clinical usefulness and limitation of these tests have not been examined in elderly patients with dementia. We performed the repetitive saliva swallowing test (RSST) and the simple swallowing provocation test (SSPT) in 37 elderly inpatients (81.8±1.2 years old). Simultaneously, cognitive and verbal communication ability were assessed by the Hasegawa Dementia Scale revised version (HDSR) and the Mini-Communication Test (MCT).

RSST was completed only in 22 patients (59%), whereas SSPT was successfully completed in all cases. Scores of HDSR and MCT were significantly lower in patients who were unable to cooperate with RSST compared to successful examinees (HDSR：7±1 vs 15±3, $p<0.01$；MCT：47±8 vs 81±5, $p<0.01$). Dysphagia was detected in 14 patients (64%) by RSST and 5 (14%) by SSPT. Patients with dysphagia showed significantly lower cognitive function ($p<0.05$) and verbal communication ability ($p<0.05$).

In conclusion, RSST is more sensitive to detect dysphagia in elderly patients；however, compliance with RSST is strongly influenced by the patient's cognitive function and verbal communication ability. Comprehensive geriatric assessment will help to choose an alternative test for dysphagia such as SSPT which is more specific test for aspiration pneumonia.

Key words：*Elderly, Dysphagia, Swallowing test, Cognitive function, Verbal communication ability*
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and specificity of EV were higher for those aged 60 and older than for those younger than 60; the sensitivity and specificity were 75.0% and 68.5% in the former and 47.9% and 56.0% in the latter. From ROC curve analysis, 14 mm was chosen as the cutoff value of EV for those aged 60 and older, and 16 mm was chosen as the cutoff value of EV for those younger than 60. The sensitivity and specificity of EV in diagnosing Graves' disease were not good in the total 113 untreated patients with Graves' disease studied, but the sensitivity and specificity of EV for the diagnosis of Graves' disease was good for those aged 60 and older.

No association was noted between EV and TRAb.

DISCUSSION

We demonstrated the clinical usefulness of exophthalmos measurements for the diagnosis of Graves' disease in older Japanese people. Although elderly patients with Graves' disease have been said not to have exophthalmos, they do when their EV is compared with that of similarly aged people without Graves' disease. Findings were that (1) exophthalmos has a diagnostic value for Graves' disease in those aged 60 and older, (2) no association was noted between EV and TRAb, and (3) EV changed with age in control subjects (the values were highest in those aged 20–29 and then gradually decreased with age) but did not in patients with Graves' disease.

EV changed with age in normal control subjects but not in patients with Graves' disease. The differences in EV between patients with Graves' disease and control subjects were significant in those aged 60 and older. ROC curve analysis showed that the sensitivity and specificity of EV were higher in those aged 60 and older than in those younger than 60. Exophthalmos has a diagnostic value for Graves' disease in older patients.

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Author Contributions: Takashi Nakamatsu measured exophthalmos and assayed TRAb, analyzed and interpreted the data, and prepared the manuscript. Nobuyuki Takasu assayed TRAb, analyzed and interpreted the data, and prepared the manuscript. Ken Nakachi measured TRAb, analyzed and interpreted the data, and prepared the manuscript.

Sponsor's Role: None.

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TAKO-TSUBO LEFT VENTRICULAR DYSFUNCTION CAUSED BY A FALL

To the Editor: We read with interest the systematic review of the psychological outcomes of falling by Jorstad et al.[1] Most of the physical complications resulting from falls are head injuries and fractures.[2,3] In addition, psychological problems sometimes induce physical complications. Falls can induce chest pain and heart failure. There have been some reports of patients with transient left ventricular dysfunction after emotional or physical stress, mostly in older women.[4–6] Here, we present a case of transient heart failure that occurred in an elderly woman after a fall.

An 85-year-old Japanese woman was referred to the University of Tokyo Hospital in July 2004 with gait disturbance, bradykinesia, anorexia, weakness, and dyspnea. Her daughter claimed that her gait disturbance had appeared a few months before and gradually worsened. She had fallen in her bedroom 2 days before and was not able to get up until the care worker visited her house the next morning. Fortunately, she had no fractures.

She exhibited stiffness and slowness of movement, stooped posture, and a mask-like facial expression and was disabled because of rigidity and slight tremor that were more marked in the right upper extremity. She had no dementia. Brain computed tomography revealed several small infarctions in the thalamus and basal nuclei, suggesting that her parkinsonism was due to multiple cerebral infarctions. Chest radiograph showed right pleural effusion but no cardiomegaly, and electrocardiogram (ECG) showed deep negative T-waves in leads I, AVL, AVF, and V_{2-6} (Figure 1A). Echocardiography revealed akinesis of the apical wall of the left ventricle, although the rest of the left ventricle was normokinetic (Figure 1C). Myocardial infarction was

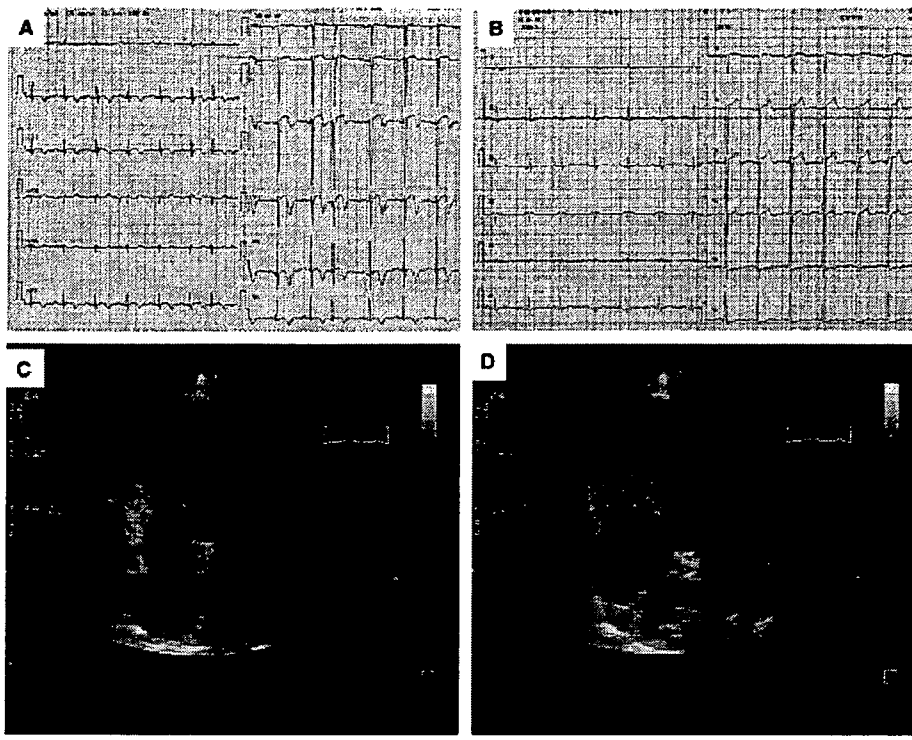

Figure 1. A. Initial electrocardiogram (ECG) showing deep negative T-waves in I, AVL, AVF, and V_{2-6}. B. ECG showing improvement of T-waves 6 months later. C and D. Echocardiography showing akinesis of the apical wall and normokinesis of the basal region of the left ventricle (C. systolic; D. diastolic).

suspected, but there was no increase in creatine kinase, troponin T, or myosin light chain. Thallium scintigraphy was performed, but no evidence of myocardial ischemia was detected. Her dyspnea improved, and the pleural effusion had disappeared by the third day without any medication.

Two weeks later, the echocardiography showed normal ventricular wall motion, although negative T-waves on the ECG remained unchanged. Finally, the ECG recovered to normal (Figure 1B) 6 months later, although she did not have any further cardiovascular medications.

After excluding drug-related cardiomyopathy, myocarditis, and myocardial infarction, based on her medical history, laboratory data, and scintigram, "tako-tsubo" (which means an octopus trap in Japanese) left ventricular dysfunction[5] was diagnosed. Transient left ventricular apical ballooning, or tako-tsubo left ventricular dysfunction, is a transient reversible cardiomyopathy with the unique feature of being induced by physical or emotional stress. The patient felt desperate when she fell and could not get up by herself, so her emotional stress may have induced this transient heart failure. Elderly women are reported to be susceptible to this disorder.[4–6] Its symptoms are similar to those of myocardial infarction, although sometimes there are none. In most cases, cardiac dysfunction is transient and recovers rapidly, although cardiogenic shock due to ventricular septal perforation[7] has been reported. Its mechanism is still unknown, although some reports suggest that reversible coronary microvascular impairment[8] or sudden surges in circulating catecholamine levels[4] are involved in this disease.

The incidence of this disease is unknown, but it is likely to be more common than generally thought. In this case, a fall resulting from parkinsonism was the cause of psychological distress, which was considered the only possible cause of tako-tsubo left ventricular dysfunction. Falls could cause psychological difficulties in older people and could be the trigger for this disorder. Fortunately the patient's heart failure was mild and did not need medical attention, but falls might cause chest pain or heart failure, as well as head injuries and fractures.

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Sponsor's Role: Not applicable.

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ALZHEIMER'S DISEASE AND MEDICAL DISEASE CONDITIONS: A PROSPECTIVE COHORT STUDY

To the Editor: Patients with early Alzheimer's disease (AD) represent a heterogeneous cohort, with some patients progressing faster to end-stage dementia and some others progressing much more slowly (Table 1). It has been postulated that various factors such as Mini-Mental State Examination (MMSE) score on initial presentation, educational level, age of onset of AD, female sex, poor performance on activities of daily living, family history, and presence of psychiatric symptoms explain this difference.[1,2] A prospective cohort study was designed to study the coexisting medical diseases at various stages of AD and to determine whether they have any effect on the progression of AD.

METHODS

A cohort of community-residing elderly persons aged 60 to 85 who fulfilled the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition,* (DSM-IV) criteria for primary degenerative dementia of the Alzheimer's type[3] and National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association criteria[4] for probable AD at baseline were followed longitudinally at intervals of 3 to 4 years. Medical, neurological, psychiatric, psychometric, and neuroradiological evaluations were conducted at baseline to exclude patients with other dementing illnesses. The Global Deterioration Scale (GDS)[5] and the MMSE were used to assess the cognitive and functional capacity at all evaluations. Further evaluations were not performed once the person reached the final, most severe stage of AD (GDS 7). The course of dementia was considered rapid if GDS 7 was reached within 4 years of the time of baseline evaluation.[6]

Criteria for exclusion at baseline included history of head trauma; seizures or other neurological disorders; mental retardation; a diagnosis of multiinfarct dementia; significant alcohol abuse; schizophrenia, depression, or major affective disorders; and cardiac, pulmonary, vascular, metabolic, or hematological conditions or other impairments of sufficient severity to adversely affect cognition or functioning.

The medical disease conditions (MDCs) were categorized as cardiovascular, endocrine, respiratory, nervous system (except dementia), hematological, neoplastic, gastrointestinal, dermatological and connective tissue disorders, allergic, history of surgeries, injuries and fractures, eye and ear, genitourinary and gynecological, and musculoskeletal disorders. The MDCs were reviewed at each visit, and their effect on the course of AD was investigated.

Table 1. Comparison Between Groups Depending on the Rapidity of Course of Dementia

	Overall (n = 40)		Faster Course (n = 28)		Slower Course (n = 12)	
Variable	Baseline	Final	Baseline	Final	Baseline	Final
Number of medical diseases, mean ± SD	5.1 ± 3.2	7.9 ± 3.9	6.0 ± 2.5*	8.1 ± 3.6	3.9 ± 3.2	7.8 ± 4.1
Geriatric Depression Scale score, mean ± SD	4.8 ± 0.9	7.0 ± 0.0	4.9 ± 0.9	7.0 ± 0.0	4.8 ± 0.8	7.0 ± 0.0
Mini-Mental State Examination score, mean ± SD, mean ± SD	13.2 ± 7.3	0.2 ± 0.8	13.3 ± 7.6	0.0 ± 0.0	13.0 ± 7.1	0.2 ± 0.9
Age, mean ± SD	69.9 ± 3.2	75.1 ± 5.8	70.6 ± 3.8	73.8 ± 3.9	68.9 ± 3.0	75.8 ± 4.2
Nursing home residence, n (%)	0 (0)	29 (73)	0 (0)	20 (71)	0 (0)	9 (75)
Peripheral vascular disease, n (%)	10 (25)	11 (28)	10 (36)*	10 (36)*	0 (0)	1 (8)
Atherosclerotic heart disease, n (%)	12 (30)	17 (43)	11 (39)*	13 (46)	1 (8)	4 (33)
Pressure mellitus, n (%)	10 (25)	11 (28)	8 (29)	8 (29)	2 (17)	3 (25)
Hypertension, n (%)	23 (58)	27 (68)	18 (64)	20 (71)	5 (42)	7 (58)
Pressure ulcer, n (%)	0 (0)	15 (38)	0 (0)	11 (39)	0 (0)	4 (33)
Contracture, n (%)	0 (0)	8 (20)	0 (0)	6 (21)	0 (0)	2 (17)
Hip fracture, n (%)	0 (0)	10 (25)	0 (0)	8 (29)	0 (0)	2 (17)

*$P < .05$ for differences between faster and slower progression of Alzheimer's disease.
SD = standard deviation.

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ORIGINAL ARTICLE

Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras–MAPK signaling in human cancer cells

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The induction of senescence-like growth arrest has emerged as a putative contributor to the anticancer effects of chemotherapeutic agents. Clinical trials are underway to evaluate the efficacy of inhibitors for class I and II histone deacetylases to treat malignancies. However, a potential antiproliferative effect of inhibitor for Sirt1, which is an NAD^+-dependent deacetylase and belongs to class III histone deacetylases, has not yet been explored. Here, we show that Sirt1 inhibitor, Sirtinol, induced senescence-like growth arrest characterized by induction of senescence-associated β-galactosidase activity and increased expression of plasminogen activator inhibitor 1 in human breast cancer MCF-7 cells and lung cancer H1299 cells. Sirtinol-induced senescence-like growth arrest was accompanied by impaired activation of mitogen-activated protein kinase (MAPK) pathways, namely, extracellular-regulated protein kinase, c-*jun* N-terminal kinase and p38 MAPK, in response to epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I). Active Ras was reduced in Sirtinol-treated senescent cells compared with untreated cells. However, tyrosine phosphorylation of the receptors for EGF and IGF-I and Akt/PKB activation were unaltered by Sirtinol treatment. These results suggest that inhibitors for Sirt1 may have anticancer potential, and that impaired activation of Ras–MAPK pathway might take part in a senescence-like growth arrest program induced by Sirtinol.
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Keywords: Sirt1; Sirtinol; cellular senescence; Ras; MAPK; Akt/PKB

Introduction

Cellular senescence is a state with permanent loss of replicative capability even upon mitogenic stimuli. Cellular senescence is characterized by phenotypic alterations including induction of senescence-associated β-galactosidase (SA-β-gal), a large and flat cell morphology and increased expression of plasminogen activator inhibitor 1 (PAI-1) (Goldstein *et al.*, 1994; Dimri *et al.*, 1995).

Immortalization, an escape from the replicative senescence program, is a necessary step for neoplastic transformation of cells. Hence, transformed cells can bypass the replicative senescence program. However, cancer and leukemia cells still retain the capacity to undergo premature senescence in response to various stimuli. Senescent normal human fibroblasts usually exhibit G1 cell cycle arrest. However, polyploidy and multinucleation are also associated with replicative senescence and premature senescence in various cell types, including human normal endothelial cells (Aviv *et al.*, 2001; Wagner *et al.*, 2001) and breast cancer MCF-7 cells (Kim *et al.*, 2003).

Anticancer chemotherapeutic agents and ionizing radiation have been shown to cause senescence-like growth arrest in human cancer cells *in vitro* and *in vivo* (Han *et al.*, 2002; Schmitt *et al.*, 2002; te Poele *et al.*, 2002; Shay and Roninson, 2004). Induction of SA-β-gal staining by chemotherapy was observed *in vivo* in cancer and lymphoma cells in rodents (Elmore *et al.*, 2002; Roninson, 2002; Schmitt *et al.*, 2002; Christov *et al.*, 2003) and in patients with breast cancer (te Poele *et al.*, 2002). Thus, senescence-like growth arrest has been proposed to be a putative determinant of *in vivo* tumor response to chemotherapeutic agents and ionizing radiation (Mathon and Lloyd, 2001; Wang *et al.*, 2003; Ben-Porath and Weinberg, 2004; Kahlem *et al.*, 2004; Pelicci, 2004; Sharpless and DePinho, 2004; Shay and Roninson, 2004).

Sirt1 is a mammalian NAD^+-dependent deacetylase that belongs to class III histone deacetylases (HDACs) (Imai *et al.*, 2000; Landry *et al.*, 2000; Blander and Guarente, 2004). Sir2, yeast homologue of Sirt1, is involved in a number of cellular processes including gene silencing at telomere and mating loci, DNA repair, recombination and aging. Recent studies demonstrated that Sirt1 plays an important role in the regulation of cell fate and stress response in mammalian cells. Sirt1 promotes cell survival by inhibiting apoptosis or cellular senescence induced by stresses including DNA damage

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and oxidative stress. An increasing number of proteins have been identified as substrates of Sirt1, including p53 (Luo *et al.*, 2001; Vaziri *et al.*, 2001; Langley *et al.*, 2002), FOXO transcription factors (Brunet *et al.*, 2004; Daitoku *et al.*, 2004; Motta *et al.*, 2004; van der Horst *et al.*, 2004), peroxisome proliferator-activated receptor-γ (Picard *et al.*, 2004) and Ku70 (Cohen *et al.*, 2004).

Sirt1 deacetylase, a member of the class III HDAC family, exhibits distinct biochemical characteristics from conventional class I and class II HDACs. Inhibitors for class I and class II HDACs, such as trichostatin A and its derivatives, do not inhibit the deacetylating activity of Sirt1 (Imai *et al.*, 2000). Conversely, specific inhibitors for Sirt1 such as Sirtinol do not inhibit class I and class II HDACs, either (Bedalov *et al.*, 2001; Grozinger *et al.*, 2001).

Class I and class II HDAC inhibitors exhibit antiproliferative effects in human cancer cells (Rosato and Grant, 2004; Vanhaecke *et al.*, 2004; Vigushin and Coombes, 2004). The efficacy of class I and class II HDAC inhibitors for treatment of patients with cancer or leukemia has been examined in phase I and II clinical trials (Piekarz *et al.*, 2001; Sandor *et al.*, 2002; Kelly *et al.*, 2003; McLaughlin and La Thangue, 2004; Piekarz and Bates, 2004). However, the effects of inhibitor for Sirt1 or class III HDACs on cell growth have not yet been investigated. Here, we show that Sirtinol, a specific inhibitor for Sirt1, induced senescence-like growth arrest in human breast cancer MCF-7 and lung cancer H1299 cells, and that Sirtinol-induced senescence-like growth arrest was accompanied by blunted activation of Ras–mitogen-activated protein kinase (MAPK) pathways in response to growth factors.

Results

Sirtinol, Sirt1 inhibitor, induced senescence-like growth arrest in human MCF-7 and H1299 cells

MCF-7 and H1299 cells were exposed to Sirtinol (100 μM) for 24 h; then Sirtinol was removed from the culture media. Treatment with Sirtinol inhibited cell growth in both MCF-7 and H1299 cells (Figure 1a and b). The inhibition of cell growth was persistent and observed up to 9 days after Sirtinol withdrawal. These results suggest that Sirtinol caused a sustained growth arrest. This was supported by reduced incorporation of BrdU in Sirtinol-treated MCF-7 and H1299 cells at 10 days after the addition of Sirtinol, as compared with untreated cells (Figure 1c and d).

We examined the effects of Sirtinol treatment on SA-β-gal activity and the expression of PAI-1, characteristic features of senescence-like growth arrest. Sirtinol treatment increased SA-β-gal-positive cells in a dose-dependent manner 10 days after the addition of Sirtinol in both MCF-7 and H1299 cells (Figures 2 and 3a), but the extent of SA-β-gal induction was relatively smaller in H1299 than in MCF-7 cells. Only a small number of MCF-7 and H1299 cells were SA-β-gal-positive when untreated. Enlarged, flattened morphology was

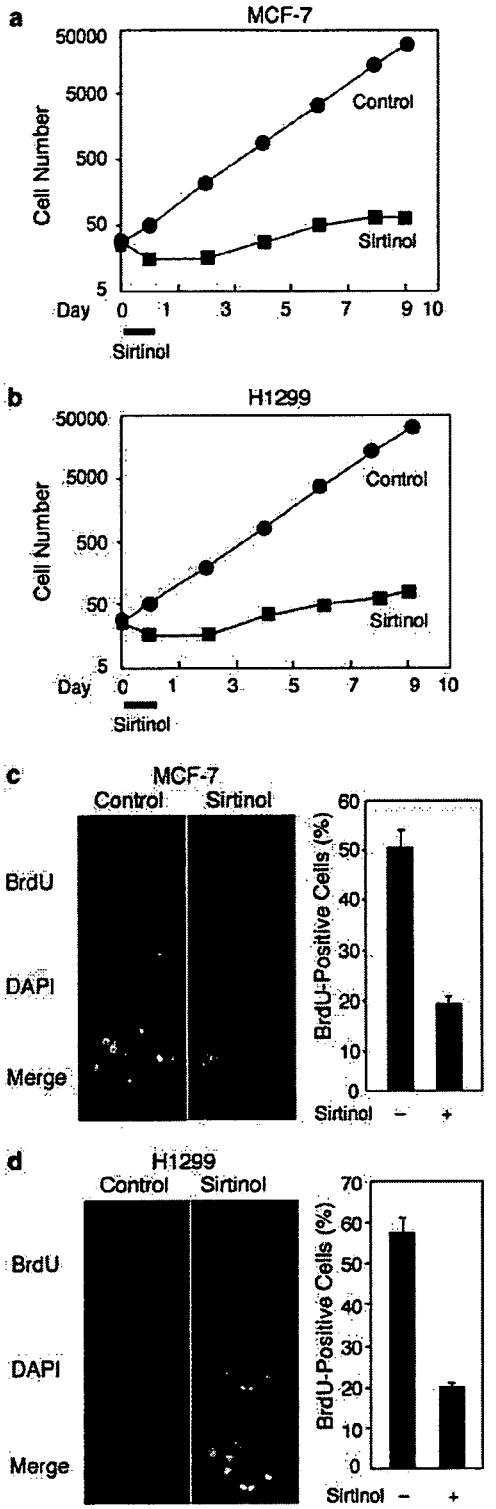

Figure 1 Effects of Sirtinol on cell growth and BrdU incorporation. MCF-7 (**a**) and H1299 (**b**) cells were treated with Sirtinol (100 μM) for 24 h. At 24 h after the addition of Sirtinol, Sirtinol was removed from the media, and then the cells were cultured in inhibitor-free complete media. (**c**, **d**) BrdU incorporation was evaluated at 10 days after the addition of Sirtinol (100 μM). BrdU incorporation was decreased in Sirtinol-treated MCF-7 (**c**) and H1299 (**d**) cells compared with untreated cells (Control).

observed in Sirtinol-treated MCF-7 cells and, to a lesser extent, in Sirtinol-treated H1299 cells, as compared with untreated cells. Sirtinol treatment also resulted in increased expression of PAI-1 in both MCF-7 and H1299 cells (Figure 3b). β-Actin expression, however, was not affected by Sirtinol.

Treatment with Splitomicin, another specific inhibitor for Sirt1, for 24 h also led to the induction of SA-β-gal staining in a dose-dependent manner (Figure 3a). However, greater concentrations of Splitomicin appeared to be required to induce SA-β-gal, as compared with Sirtinol.

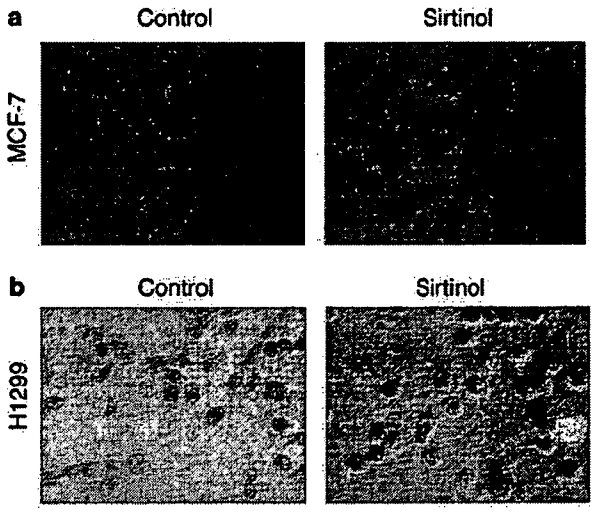

Figure 2 SA-β-gal staining in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), MCF-7 (**a**) and H1299 (**b**) cells were stained for SA-β-gal. Sirtinol treatment increased SA-β-gal-positive cells in MCF-7 and H1299 cells. In addition, the number of multinucleated cells was increased in Sirtinol-treated MCF-7 cells, but not in Sirtinol-treated H1299 cells, compared with untreated (Control) cells. Arrowheads denote multinucleated cells in Sirtinol-treated MCF-7 cells.

Colony formation assay also revealed that both Sirtinol and Splitomicin elicited antiproliferative effects in MCF-7 and H1299 cells in a dose-dependent manner (Figure 4). Sirtinol inhibited colony formation at concentrations of 33 μM and higher in MCF-7 and H1299 cells. On the other hand, 33 μM Splitomicin failed to decrease the number of colonies, but Splitomicin at 100 and 333 μM effectively inhibited colony formation in MCF-7 and H1299 cells.

Senescence-like growth arrest by Sirt1 inhibition was further corroborated by experiments using short interfering RNA (siRNA). Gene knockdown of Sirt1 by siRNA resulted in induction of SA-β-gal staining, large and flat cell morphology, decreased BrdU incorporation and increased PAI-1 expression in both MCF-7 and H1299 cells, as compared with control siRNA (Figure 5). Moreover, Sirt1 inhibition by Sirtinol, Splitomicin or siRNA also induced senescence-like phenotype in human diploid fibroblasts, WI-38 and IMR-90 cells, reflected by induction of SA-β-gal staining, and enlarged and flattened cell morphology (Supplementary Figure 1).

In Sirtinol-treated MCF-7 cells, the number of multinucleated cells was increased compared with untreated cells (Figure 2), but multinucleated cells were not found in H1299 cells regardless of whether treated with or without Sirtinol. Consistent with these observations, flow cytometric analysis revealed that the substantial cell population of Sirtinol-treated MCF-7 cells exhibited DNA content over 4*N*, indicative of polyploidy (Figure 6a). Polyploidy fraction estimated by the ratio

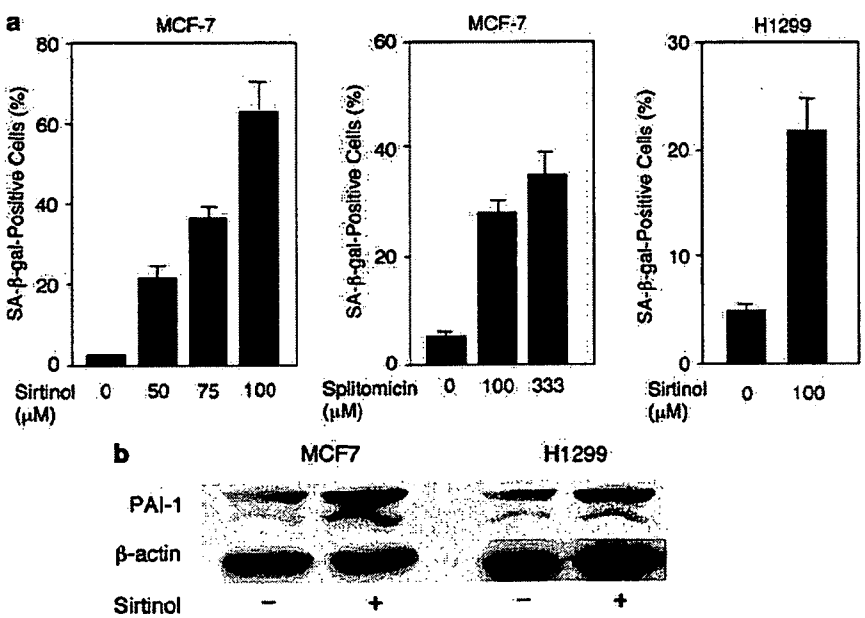

Figure 3 Effects of Sirtinol and Splitomicin on SA-β-gal activity and PAI-1 expression. (**a**) SA-β-gal-positive cells were counted 10 days after the addition of indicated concentrations of Sirtinol or Splitomicin in MCF-7 and H1299 cells. Treatment with Sirtinol and Splitomicin increased SA-β-gal-positive cells in a dose-dependent manner. (**b**) Sirtinol treatment resulted in the increased expression of PAI-1 as compared with untreated cells.

of cell population with DNA content of over 4*N* to that with over 2*N* was greater in Sirtinol-treated MCF-7 cells than in untreated cells (Figure 6a, right panel). In contrast, Sirtinol-treated H1299 cells were cell cycle arrested at G1 (Figure 6b). There was little, if any, increase in polyploidy fraction by Sirtinol in H1299 cells (control: 0.6%; Sirtinol: 1.6%).

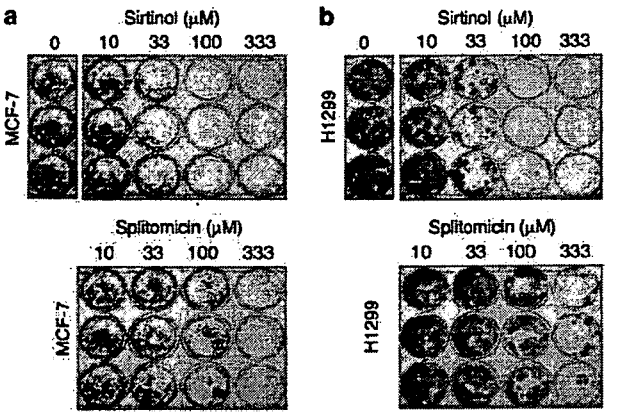

Figure 4 The effects of Sirtinol and Splitomicin on colony formation. MCF-7 (a) and H1299 (b) cells were inoculated onto 12-well plates at the density of 500 cells/well, and treated with the indicated concentrations of Sirtinol or Splitomicin for 24 h. After withdrawal of the Sirt1 inhibitors, the cells were cultured for 14 days. Both Sirtinol and Splitomicin inhibited colony formation in a dose-dependent manner.

In cellular senescence, induction of p53, dephosphorylation of Rb and increased expression of cyclin-dependent kinase inhibitors such as p16, p21 and p27 have been shown to be involved (Serrano *et al*., 1997; Collado *et al*., 2000; Alexander and Hinds, 2001; Beausejour *et al*., 2003; Jirawatnotai *et al*., 2003; Mallette *et al*., 2004). We found that phosphorylated Rb was decreased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells, while the protein expression of Rb was unaltered (Figure 7a and b). p27 expression was induced in Sirtinol-treated MCF-7 and H1299 cells (Figure 7f and g), while β-actin expression was unaltered. However, the mRNA level of p27 was not increased by Sirtinol treatment in MCF-7 and H1299 cells at both 3 and 10 days after the addition of Sirtinol, while tamoxifen and serum starvation upregulated the p27 mRNA level in MCF-7 and H1299 cells, respectively (Supplementary Figure 2). In contrast, p21 was not increased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells. p16 was not induced in Sirtinol-treated H1299 cells (Figure 7g). MCF-7 and H1299 cells are deficient in p16 and p53, respectively. On the other hand, treatment with tamoxifen (1 μM) for 24 h increased protein expression of p21 and p27 in MCF-7 cells, and serum starvation for 24 h induced p16, p21 and p27 expression in H1299 cells. Neither expression nor acetylation of p53 was upregulated by Sirtinol

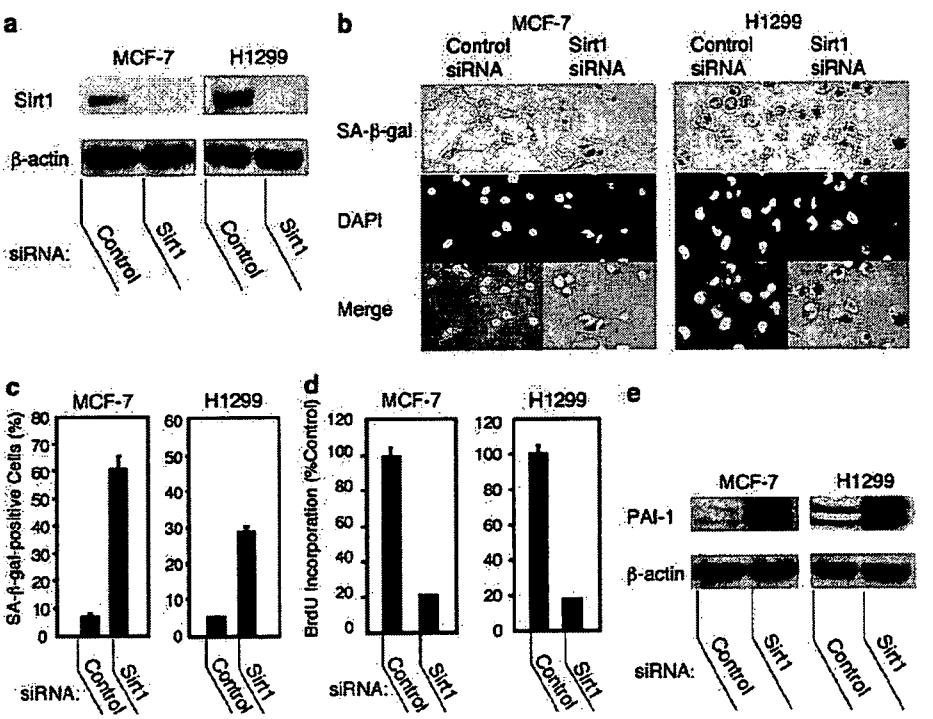

Figure 5 Gene knockdown of Sirt1 by siRNA induced senescence-like phenotype. MCF-7 and H1299 cells were treated with siRNA for Sirt1 or control siRNA. (a) At 3 days after the transfection, immunoblot analysis revealed that Sirt1 siRNA effectively reduced Sirt1 expression in both MCF-7 and H1299 cells. (b, c) At 10 days after the transfection, SA-β-gal-positive cells were significantly increased in Sirt1 siRNA-treated cells compared with control siRNA-treated cells. (d) BrdU incorporation was decreased in Sirt1 siRNA-treated cells compared with control siRNA-treated cells at 10 days after the transfection. (e) Sirt1 siRNA increased PAI-1 protein expression compared with control siRNA at 10 days after the transfection.

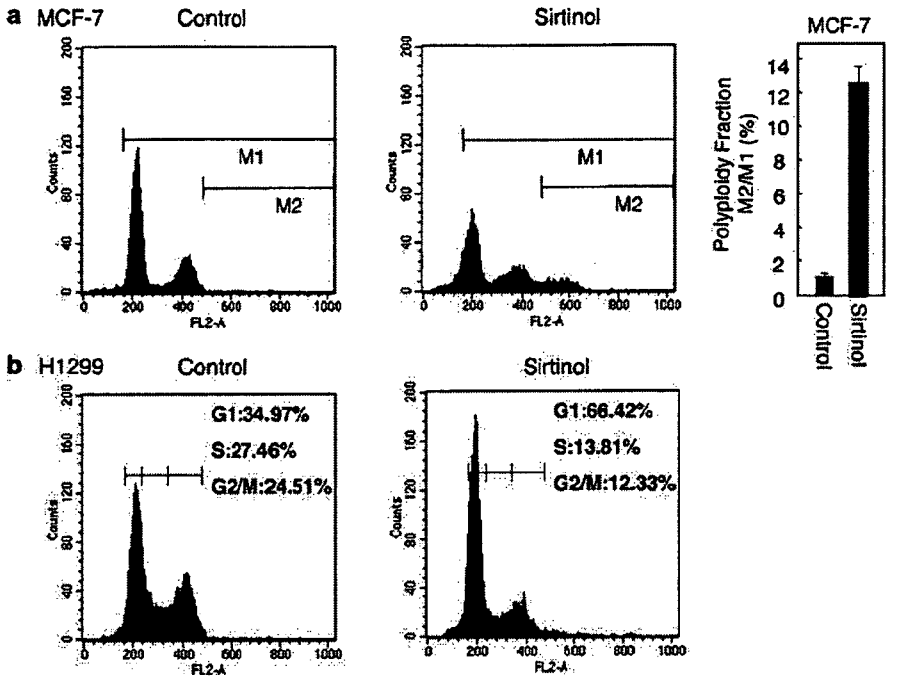

Figure 6 Flow cytometric analysis of Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), the cell cycle of MCF-7 (a) and H1299 (b) cells was analysed by flow cytometry. In Sirtinol-treated MCF-7 cells, polyploidy fraction (M2/M1; cell population with DNA content of over 4*N* normalized to that with DNA content of over 2*N*) was increased compared with untreated MCF-7 cells (Control). Sirtinol-treated H1299 cells exhibited G1 cell cycle arrest (b).

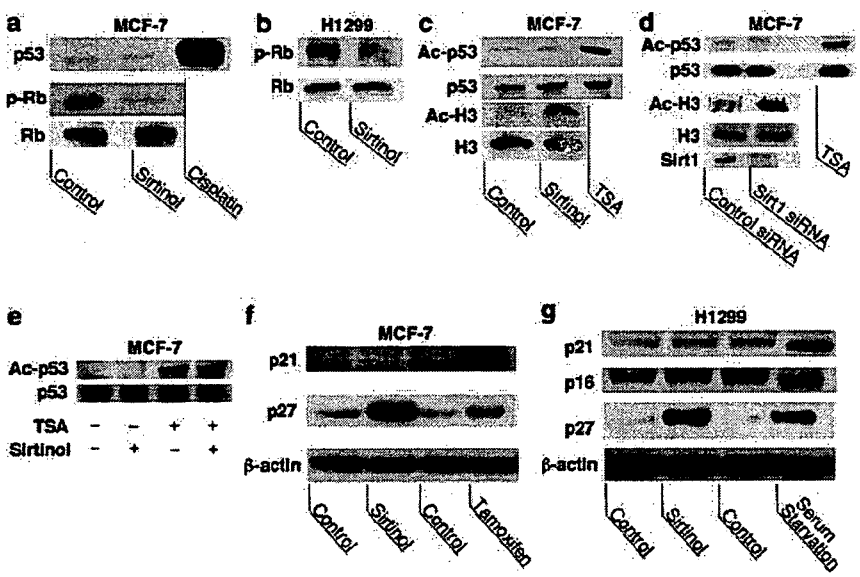

Figure 7 p53, Rb and cyclin-dependent kinase inhibitors in Sirtinol-treated cells. (a, b) At 10 days after the addition of Sirtinol (100 μM), phosphorylated Rb (p-Rb) was decreased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells. (a, c, d) The expression and acetylation of p53 were not increased by Sirtinol (a, c) or siRNA for Sirt1 (d) in MCF-7 cells. However, a robust increase in expression and acetylation of p53 (Ac-p53) were found in MCF-7 cells when treated with cisplatin (40 μM) for 18 h and trichostatin A (TSA, 5 μM) for 4 h, respectively. In contrast, Sirtinol (100 μM) treatment and siRNA for Sirt1 increased acetylated histone H3 (Ac-H3), while the abundance of histone H3 was unaltered (c, d). (e) MCF-7 cells were treated with or without Sirtinol (100 μM) in the presence or absence of trichostatin A (TSA, 0.5 μM) for 24 h. Sirtinol enhanced trichostatin A-induced acetylation of p53. p53 expression was not altered by Sirtinol or trichostatin A. (f, g) p21 expression was not increased by Sirtinol treatment in MCF-7 and H1299 cells. p16 expression was not induced by Sirtinol treatment in H1299 cells. In contrast, the expression of p27 was increased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells.

or siRNA for Sirt1 in MCF-7 cells that harbor wild-type p53 (Figure 7c and d), while acetylation of histone H3 was increased by Sirtinol and siRNA for Sirt1. However, cisplatin and trichostatin A, class I and class II HDAC inhibitor, caused robust induction of p53 and acetylation of p53 in MCF-7 cells, respectively (Figure 7a and c–e). Although Sirtinol alone did not increase p53 acetylation, Sirtinol enhanced p53 acetylation in the presence of trichostatin A (Figure 7e). These results are consistent with previous observations that inhibition of Sirt1 by itself did not induce p53 acetylation in the absence of other stimulus, while DNA damage- or oxidative stress-induced p53 acetylation was accentuated by Sirt1 inhibition (Luo *et al.*, 2001; Vaziri *et al.*, 2001; Langley *et al.*, 2002; Cheng *et al.*, 2003).

Senescence-like growth arrest was accompanied by attenuated activation of MAPK pathways in response to growth factors

We examined the activation status of signaling pathways of MAPKs and Akt/PKB in response to growth factors, epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I). When untreated with Sirtinol, upon exposure to EGF or IGF-I, robust phosphorylation of extracellular-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK/SAPK, also termed stress-activated protein kinase) and p38 MAPK was observed in MCF-7 and H1299 cells. By contrast, in Sirtinol-treated senescent MCF-7 and H1299 cells at 10 days after the addition of Sirtinol (100 μM), basal (unstimulated) phosphorylation of ERK, JNK/SAPK and p38 MAPK was reduced compared with untreated cells (Figure 8). In addition, EGF- or IGF-I-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was attenuated in MCF-7 and H1299 cells at 10 days after the addition of Sirtinol, compared to untreated cells.

Reduced activation of ERK, JNK/SAPK and p38 MAPK was corroborated by the phosphorylation status of the endogenous substrates of these MAPKs. Basal (unstimulated) as well as EGF- or IGF-I-stimulated phosphorylation of Elk-1, c-Jun and ATF-2 was also decreased in Sirtinol-treated senescent MCF-7 and H1299 cells at 10 days after the addition of Sirtinol, compared with untreated cells (Figure 9). The protein expression of ERK, JNK/SAPK, p38 MAPK, Elk-1, c-Jun and ATF-2 did not differ between Sirtinol-treated and untreated MCF-7 and H1299 cells.

However, at 3 days after the addition of Sirtinol, unlike at 10 days after the inhibitor addition, EGF-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was not attenuated in MCF-7 cells, compared with untreated cells (Figure 8d). These results suggest that attenuated MAPK pathways may be a consequence, rather than a cause, of Sirtinol-induced commitment of senescence-like growth arrest.

In contrast, tyrosine phosphorylation of the receptors for EGF and IGF-I by their ligands was not altered by Sirtinol treatment in MCF-7 and H1299 cells at 10 days after the addition of Sirtinol (Figure 10). The expression of EGF receptor and IGF-I receptor did not differ between Sirtinol-treated and untreated cells. These findings suggest that the defects responsible for impaired activation of MAPKs may exist at the level(s) of postreceptor signaling cascades.

Ras plays a critical role in growth factor-stimulated activation of MAPK pathways. Active Ras was markedly increased by EGF in untreated MCF-7 and H1299 cells. In Sirtinol-treated senescent MCF-7 and H1299 cells, however, the basal (unstimulated) level of active Ras was reduced compared with untreated cells, and EGF failed to increase active Ras (Figure 11a). Consistent with defective activation of Ras, basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Raf-1, MEK, SEK1/MKK4 and MKK7 was attenuated in Sirtinol-treated cells relative to untreated cells (Figure 11b). However, no difference was found between Sirtinol-treated and untreated MCF-7 and H1299 cells in the protein expression of Ras, Raf-1, MEK, SEK1/MKK4 and MKK7.

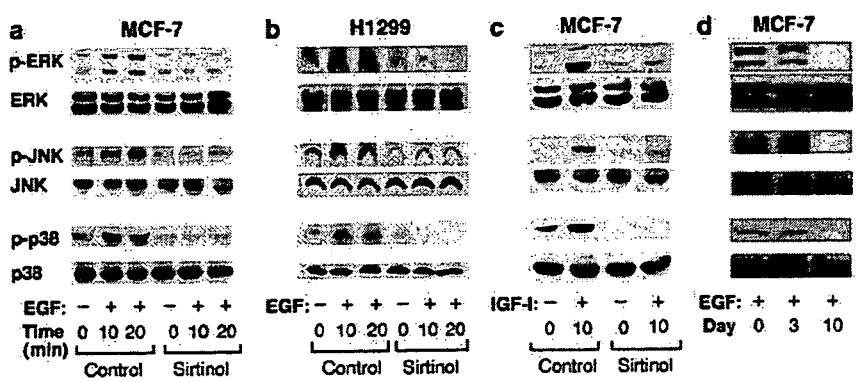

Figure 8 Growth factor-stimulated phosphorylation of MAPKs in Sirtinol-treated cells. (a–c) At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (a, b), or to IGF-I (100 ng/ml) for 10 min (c). In untreated (Control) MCF-7 and H1299 cells, marked phosphorylation of ERK, JNK/SAPK and p38 MAPK was induced by EGF or IGF-I. In Sirtinol-treated MCF-7 and H1299 cells, basal (unstimulated) as well as EGF- and IGF-I-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was decreased compared with untreated cells. (d) At 0, 3 and 10 days after the addition of Sirtinol (100 μM), MCF-7 cells were stimulated with EGF (50 ng/ml) for 20 min. EGF-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was markedly impaired at 10 days, but preserved at 3 days after the addition of Sirtinol.

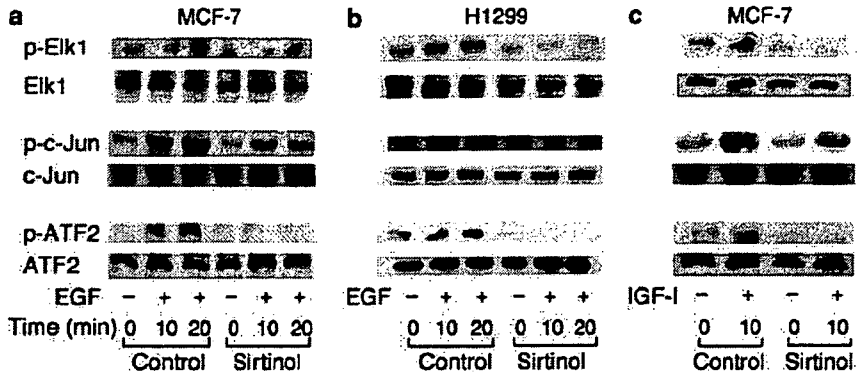

Figure 9 Growth factor-stimulated phosphorylation of Elk1, c-Jun and ATF2. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (a, b) or to IGF-I (100 ng/ml) for 10 min (c). In untreated (Control) MCF-7 and H1299 cells, marked phosphorylation of Elk1, c-Jun and ATF2 was induced by EGF and IGF-I. In Sirtinol-treated MCF-7 and H1299 cells, both basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Elk1, c-Jun and ATF2 were decreased compared with untreated cells.

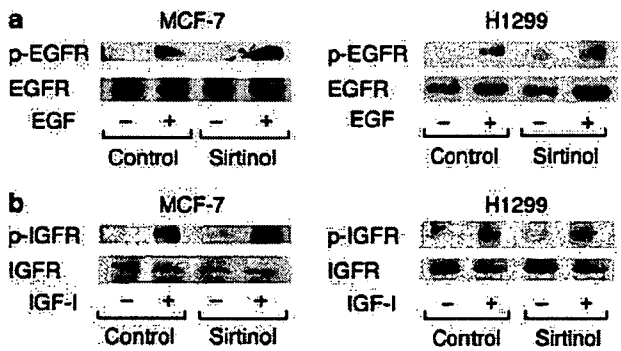

Figure 10 Growth factor-stimulated phosphorylation of EGF receptor and IGF-I receptor in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) (a) or IGF-I (100 ng/ml) for 2 min (b). There was no difference in tyrosine phosphorylation and protein expression of EGF receptor (EGFR) and IGF-I receptor (IGFR) between Sirtinol-treated and untreated (Control) MCF-7 and H1299 cells.

In contrast to the Ras–MAPK pathway, EGF- or IGF-I-induced as well as basal (unstimulated) phosphorylation of Akt/PKB was not decreased in Sirtinol-treated senescent MCF-7 and H1299 cells compared with untreated cells at 10 days after Sirtinol addition (Figure 12). The expression of Akt/PKB was not altered by Sirtinol treatment, either.

Discussion

We found that Sirt1 inhibition by specific inhibitors, Sirtinol and Splitomicin, and siRNA caused senescence-like growth arrest in human cancer MCF-7 and H1299 cells, as judged by SA-β-gal staining, PAI-1 expression, BrdU incorporation, flattened and enlarged morphology of the cells and flow cytometric analysis (Figures 1–6). Sirtinol-induced senescence-like growth arrest was accompanied by attenuated responses to growth factors in terms of activation of Ras–MAPKs (Figures 8, 9 and 11). By contrast, phosphorylation (activation) of EGF receptor, IGF-I receptor and Akt/PKB by growth factors was not affected in Sirtinol-treated senescent MCF-7 and H1299 cells (Figures 10 and 12).

Consistent with impaired activation of MAPKs, EGF- and IGF-I-stimulated phosphorylation of downstream targets, Elk-1, c-Jun and ATF-2, was also reduced (Figure 9). A hallmark feature of senescent cells is unresponsiveness to mitogenic stimuli in terms of induction of c-*fos* as well as cell proliferation. Previous studies in senescent human diploid fibroblasts showed that induction of c-*fos* (Seshadri and Campisi, 1990) and activation of Elk-1 (Tresini *et al.*, 2001) and MEK–ERK (Torres *et al.*, 2003) in response to growth factors are impaired. Transcriptional activity of Elk-1 regulates the induction of c-*fos*, an immediate early gene. Thus, our results of attenuated activation of Ras–MEK–ERK–Elk-1 in Sirtinol-treated senescent cancer cells are in agreement with previous findings in senescent human fibroblasts.

Our results indicate that the signaling defect in Sirtinol-treated cells is specific for MAPK pathways and that the PI3-K–Akt/PKB pathway is preserved. Ras is a key regulator of MAPK pathways (Lange-Carter and Johnson, 1994). However, Ras does not play a major role in activation of the PI3-K–Akt/PKB pathway (Sakaue *et al.*, 1995; Gnudi *et al.*, 1997; Klesse *et al.*, 1999). Our results showed that active, GTP-bound Ras was reduced in Sirtinol-treated cancer cells compared with untreated cells (Figure 11a). The present data, therefore, suggest that reduced activation of Ras might be involved in a specific attenuation in MAPK pathways in Sirtinol-treated senescent MCF-7 and H1299 cells.

Growth factor-initiated mitogenic signals are conveyed mainly by two major signaling cascades: Ras–ERK and PI3-K–Akt/PKB. Senescent cells remain viable and metabolically active, in spite of irreversible loss of replication capability (Roninson, 2003; Shay and Roninson, 2004). One can reasonably speculate, therefore, that the preserved PI3-K–Akt/PKB pathway might

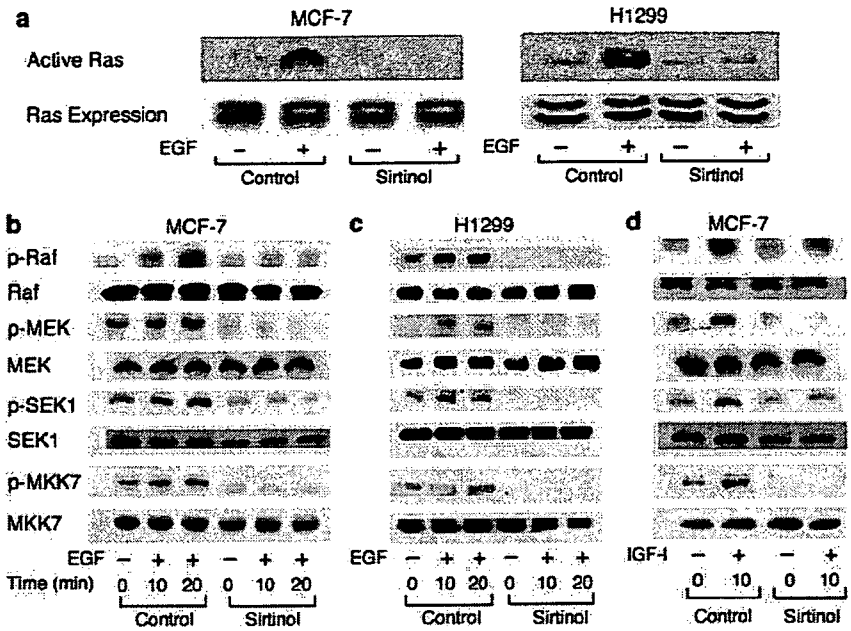

Figure 11 Activation status of Ras and its downstream signaling molecules in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min, or IGF-I (100 ng/ml) for 10 min. (**a**) Active Ras was evaluated as described in Materials and methods. In untreated (Control) MCF-7 and H1299 cells, active Ras was markedly increased by EGF treatment for 20 min. In Sirtinol-treated MCF-7 and H1299 cells, basal (unstimulated) level of active Ras was decreased compared with untreated cells, and EGF failed to increase active Ras. (**b–d**) In untreated (Control) MCF-7 and H1299 cells, EGF and IGF-I induced robust phosphorylation of Raf, MEK, SEK1/MKK4 and MKK7. However, in Sirtinol-treated MCF-7 and H1299 cells, both basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of these molecules were decreased compared with untreated (Control) cells.

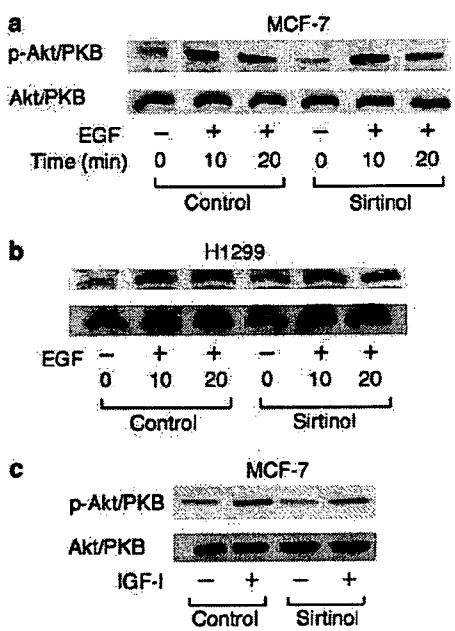

Figure 12 Growth factor-stimulated Akt/PKB phosphorylation in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (**a**, **b**) or to IGF-I (100 ng/ml) for 10 min (**c**). No difference was found in basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Akt/PKB between Sirtinol-treated and untreated (Control) MCF-7 and H1299 cells.

contribute to cell viability and metabolic activities in Sirtinol-treated cells, because the PI3-K–Akt/PKB pathway plays critical roles in cell survival and regulation of metabolism.

p53, Rb and cyclin-dependent kinase inhibitors such as p16, p21 and p27 have been recognized as key mediators of cellular senescence (Serrano *et al.*, 1997; Collado *et al.*, 2000; Alexander and Hinds, 2001; Beausejour *et al.*, 2003; Jirawatnotai *et al.*, 2003; Mallette *et al.*, 2004). Our results showed that hypophosphorylation of Rb and increased expression of p27 were associated with Sirtinol-induced senescence-like growth arrest in MCF-7 and H1299 cells (Figure 7). In addition to regulation at transcriptional level, increased p27 expression may result from reduced protein degradation through a ubiquitin–proteasome system (Carrano *et al.*, 1999). Since Sirt1 is an HDAC, it is possible that Sirt1 inhibition may directly modulate p27 transcription. However, our finding of unaltered p27 mRNA in Sirtinol-treated cells (Supplementary Figure 2) suggests that decreased protein degradation of p27, rather than increased transcription, may contribute to increased p27 protein expression in Sirtinol-treated cells. Thus, our data argue against a direct effect of Sirtinol on transcription of p27.

On the other hand, we did not find increased expression of p53, p21 and p16 in Sirtinol-treated MCF-7 and H1299 cells. It is important to note, however, that previous studies showed that premature senescence can be readily induced independent of p53,