であるが，薬剤性間質性肺炎には特異的な所見が少ないため，これらのみで確定診断を行うことは難しい．しかし，呼吸器感染症，腫瘍浸潤などとの鑑別には有用であり，またその病理所見を認識することにより，治療に対する反応性，予後の予測など，臨床的に重要な情報を得ることはできる．しかし，急性呼吸不全を示す症例では全身状態などから実施困難な場合も多い．

前述のように鑑別診断が重要であり，呼吸器感染症，特発性間質性肺炎，膠原病・血管炎に伴う間質性肺炎，心不全，他の薬剤による肺障害などびまん性間質性陰影を呈する他の疾患や肺塞栓血栓症などを除外することが必須である．

b 分子標的治療薬による肺障害の治療

薬剤性肺障害の治療は，第一に原因となっている薬剤投与を中止することである．しかし，従来の分子標的治療薬と比べ高頻度に薬剤性肺障害の発現が認められるmTOR（mammalian target of rapamycin）阻害薬では，比較的軽度かつ無症状の場合，投与継続が可能という従来の薬剤性肺障害とは異なるマネジメントが例外的に推奨されていることは留意するべきである．次にステロイド薬投与があげられる．呼吸不全を呈する場合など，重症度が高い症例に対してはステロイドパルス療法を積極的に考慮すべきである．ステロイド薬の効果が不十分な場合，免疫抑制薬を用いる場合もある．重症の呼吸不全例では，腫瘍の状況により人工呼吸管理の適応となる．また，ステロイド薬の無効なびまん性肺胞傷害と考えられる病態に対するポリミキシンカラムによる吸着療法の試みも報告されている[8]．一方で，薬剤性肺障害を避ける努力も重要と考えられ，各薬剤における危険因子の研究も進められている[1]［タルセバ錠 非小細胞肺癌 特定使用成績調査（全例調査）中間結果報告，中外製薬］．

c 各薬剤による肺障害

前述のように各薬剤による差異も明らかになっており，各論として紹介する．

①gefitinib，erlotinib ➡「Ⅱ章A-5」82頁参照

gefitinibは「手術不能または再発非小細胞肺癌」に対して2002年に承認

されたEGFRチロシンキナーゼ阻害薬である．gefitinibの肺障害については種々の調査が行われたが，とくに「非小細胞肺癌患者におけるgefitinib投与及び非投与での急性肺障害・間質性肺炎の相対リスク及び危険因子を検討するためのコホート内ケースコントロールスタディ」は前例のない大規模な（4,423例）プロスペクティブスタディであり，多くの知見が得られた[1]．この試験は，①進行／再発非小細胞肺癌患者のgefitinib投与例における間質性肺疾患（ILD）発症について，他の化学療法薬投与例との相対リスクを推定し，ILD発症に対する危険因子を検討する，②治療中の進行／再発非小細胞肺癌患者におけるILDの発症率を推定すること，を主要な目的として行われた．薬物動態学的特性の評価，塩基多型（SNP）との関係，蛋白発現パターンの解析等も探索的に検討された．登録から12週間追跡結果でILDを発症した例は，gefitinib群3.98%，他の化学療法群2.09%であった．①gefitinib治療，②喫煙歴有，③既存の間質性肺炎有，④非小細胞肺癌の初回診断からILD発症までの期間が6ヵ月以内，⑤PS 2以上，⑥正常肺占有率が低いこと（50%以下），⑦年齢55歳以上，⑧心血管系の合併症を有していること，がILD発症の危険因子としてあげられた．gefitinibのILD発症リスクは，調整オッズ比3.23であり，とくに治療開始後4週間のオッズ比は3.8と高かった．

ILD発症時の致死率は，gefitinib群と化学療法群とでほぼ同様（約30%）であり，①65歳以上，②喫煙，③既存の間質性肺炎有，④正常肺占有率が50%未満（治療前のCT画像から検討），⑤呼吸性移動制限領域が50%以上（治療前のCT画像から検討），が共通の予後不良因子としてあげられた．また，薬物動態と急性肺障害・間質性肺炎の発現の相関が検討され，発症前の薬物動態と急性肺障害・間質性肺炎の発現例との関連性は認められなかった．少なくとも，定常状態での薬物代謝の個体差は大きな要因ではないように推測される．

erlotinibは，gefitinibと同様に非小細胞肺癌を対象として用いられているEGFRチロシンキナーゼ阻害薬であり，2007年に承認された．erlotinibについては「タルセバ錠 非小細胞肺癌 特定使用成績調査（全例調査）」という施設を限定した全例調査が行われ，現在第3回の中間解析結果が公開されている［タルセバ錠 非小細胞肺癌 特定使用成績調査（全例調査）中間結果報

告, 中外製薬]. その概要は, 調査票が回収された4,662例の安全性解析結果から, ILD様事象が237例 (5.08%) に認められ, そのうち死亡例は65例であり, 投与開始から発現までの期間は, gefitinibと同様4週以内の発現が多い (159/237例). Cox比例ハザードモデルを用いた多変量解析により, ①PS (2～4), ②喫煙歴有, ③合併または既往歴 (間質性肺疾患有, 肺気腫またはCOPD有, 肺感染症有), ④肝障害 (合併有), ⑤非小細胞肺癌の初回診断日から投与開始までの期間 (360日未満), ⑥原疾患に対する薬物療法レジメン数が多い症例において, ILD様事象発現が有意に高いことが判明した. 現時点では, erlotinibの肺障害はgefitinibと発現頻度, 病態ともにほぼ同様のものであると考えられている.

この情報について適正使用検討委員会による評価が行われ[2], ILD様事象診断時の画像パターンが, 比較的予後良好と考えられている "faint infiltration pattern/acute HP pattern" を示した症例のうち, 約1/3が予後不良であったことが新たにわかった. このことから "faint infiltration pattern/acute HP pattern" のような画像パターンは, 本来のILDの特徴が示されている場合と, 他の画像パターンを示すILDの早期像の場合とが混在していると考えられた. したがって "faint infiltration pattern/acute HP pattern" が認められた場合には, 引き続きX線などによる注意深い経過観察が必要である.

② bortezomib ➡「Ⅱ章A-10」126頁参照

bortezomibは,「再発または難治性多発性骨髄腫」に対し2006年に承認されたプロテアソーム阻害薬であるが, わが国における肺障害が報告された[9]. 何らかの造血幹細胞移植施行が, 多変量解析でも危険因子である可能性が示唆され, 副腎皮質ステロイド薬の併用がリスク減少因子として抽出されている[10]. この肺障害については第三者評価委員会で検討され, びまん性肺胞傷害の存在が病理的に確認された. 全例に胸水貯留を認め, 心嚢水, 気道粘膜の浮腫を示唆する気管支壁の肥厚と内腔の狭小化などの特徴的な所見を呈す症例が認められた (**図22**) (ベルケイド適正使用ガイド, 2006, ヤンセンファーマ). 報告された製造販売後の特定使用成績調査中間解析では, 525例中22例 (4.19%) に間質性肺炎/肺障害が発現し, 重篤例は11例 (2.10%) であった [ベルケイド注射用3mg中間集計結果 (500例2サイクル

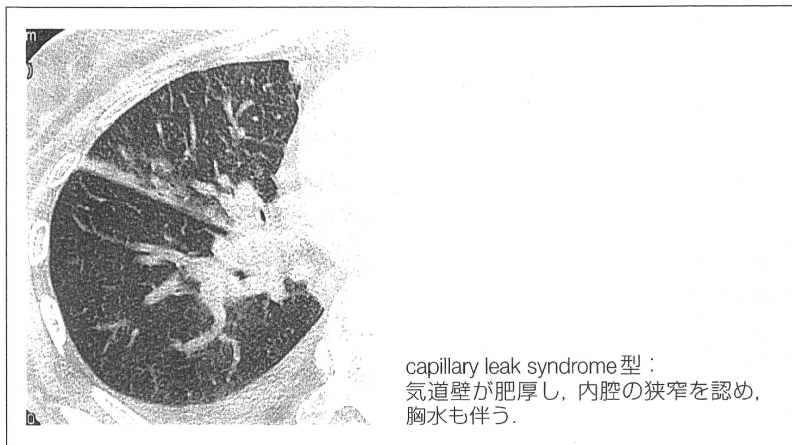


図22　bortezomibによる肺障害

（Miyakoshi S et al：Severe pulmonary complications in Japanese patients after bortezomib treatment for refractory multiple myeloma. Blood **107**：3492-3494, 2006より引用）

データを用いた集計結果），ヤンセンファーマ］．

③ imatinib ➡「Ⅱ章A-4」72頁参照

imatinibは，「慢性骨髄性白血病，KIT（CD117）陽性消化管間質腫瘍およびPhiladelphia染色体陽性急性リンパ球性白血病」に対して，2005年に承認されたbcr-ablおよびKITチロシンキナーゼ阻害薬であるが，レトロスペクティブに病態の検討が詳細になされた．推定使用者数約5,500例で，27例（0.49%）の肺障害報告があり，本有害事象による死亡症例はない[11)]．imatinibについては調査方法が異なることから，肺障害の発現頻度を他の分子標的治療薬で行われた全例調査と同列に比較することはできない．また，薬剤性肺障害の画像所見では，びまん性肺胞傷害に相当すると考えられる症例は認められず[11)]，この薬剤の肺障害の特徴と考えられる．

④ bevacizumab ➡「Ⅱ章A-1」38頁参照

bevacizumabは，「治癒切除不能な進行・再発の結腸・直腸癌」に対して2007年に承認された抗VEGFヒト化モノクローナル抗体である．特定使用成績調査（全例調査）の結果が報告されている（アバスチン点滴静注用

100mg/4mL, 400mg/16mL特定使用成績調査集計結果, 中外製薬). bevacizumabは単剤ではなく他の抗癌薬との併用で使用されることから, bevacizumab単独での肺障害の発現状況についてはこの調査からは不明であるが, 併用療法の使用実態下では, 集計対象例2,698例中10例 (0.37%) にILDが, 4例 (0.15%) にARDSが発現し, 全例が重篤であり, そのうちILDは2例が, ARDSは3例が死亡に至った. 2009年には扁平上皮癌を除く切除不能な進行・再発の非小細胞肺癌に対しても承認されたが, 肺癌患者においてはILD以外に喀血等の出血の副作用についても注意が必要である.

⑤everolimus, temsirolimus ➡「Ⅱ章A-11」136頁参照

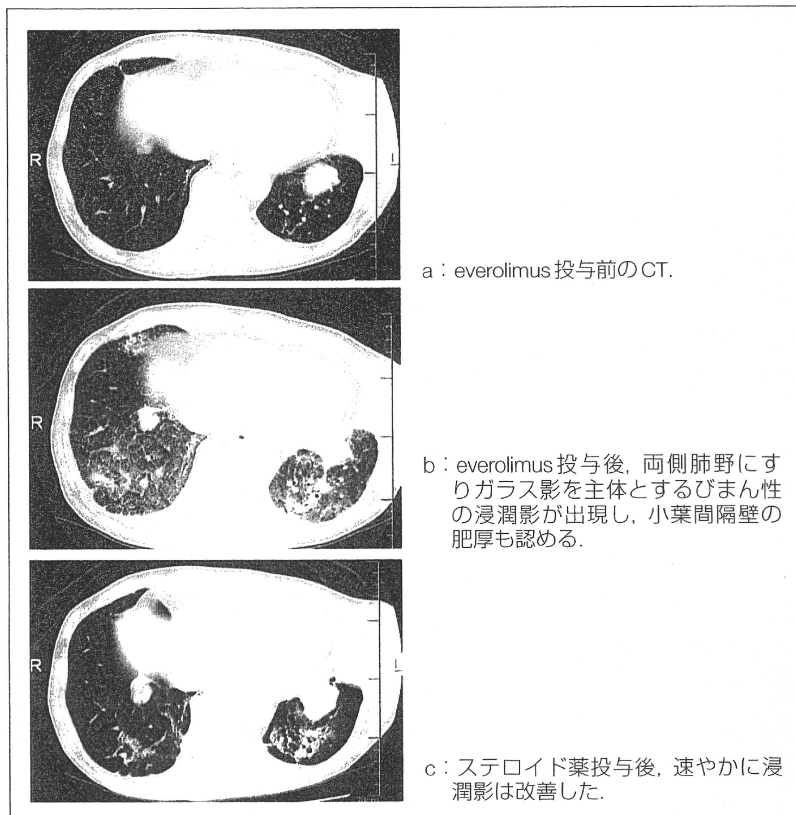
sirolimus誘導体であるeverolimusは,「根治切除不能または転移性の腎細胞癌」に対して2010年1月に承認されたmTOR阻害薬である. everolimusの腎細胞癌を対象とした日本人15例を含む第Ⅲ相国際共同試験 (RECORD-1試験) では, 副作用としてILDが11.7% (32/274例) に認められ, また現在開発段階であるが, 進行性胃癌を対象とした国内第Ⅱ相試験ではILDが15.1% (8/53例) に認められている (アフィニトール錠5mg医薬品インタビューフォーム第1版, ノバルティスファーマ). sirolimus誘導体の副作用としてILDは既知の事象である. everolimusのILD発現頻度は他の薬剤と比較して高い傾向にあるが, 無症候性に発現することがあり, 重症化することが少なく投与中止により回復し得ること, ステロイド薬に対する反応性が良好であることなどの特徴を有する. 臨床試験では症状がなく画像所見のみ認める場合 (Grade 1) には投与継続が可能となっている点が, 一般的な薬剤性肺障害に対する治療指針とは異なる. everolimus投与中にILDが発現した場合の減量・休薬基準/治療指針はILDの重症度に応じて示されており, 詳細はアフィニトール適正使用ガイドを参照されたい. everolimusによるILDのCT画像を示す (**図23**).

なお, 類薬であるtemsirolimus (mTOR阻害薬の注射薬) が最近承認され, temsirolimusにおいても同様にILDの発現に注意が必要である.

⑥その他の分子標的治療薬 ➡「Ⅱ章A-2」47頁,「Ⅱ章A-3」63頁参照

その他, 多くの分子標的治療薬の使用成績調査が全例調査として行われている.

cetuximabは,「EGFR陽性の治癒切除不能な進行・再発の結腸・直腸癌」



a：everolimus投与前のCT.

b：everolimus投与後，両側肺野にすりガラス影を主体とするびまん性の浸潤影が出現し，小葉間隔壁の肥厚も認める.

c：ステロイド薬投与後，速やかに浸潤影は改善した.

図23　everolimusによる肺障害

に対して，2008年に承認された抗ヒトEGFRモノクローナル抗体であり，「アービタックス注射液100 mg使用成績調査（全例調査）」が実施中である.市販直後調査結果（推定投与患者数3,436例）では16例のILDが報告され，全例が重篤であり，そのうち4例が死亡に至っている.これらの多くは他の抗癌薬との併用下に発現しているが，cetuximab単独投与による発現2例も含まれている（アービタックス注射液100 mg市販直後調査 副作用集計結果報告，メルクセローノ）.

表33 製造販売後調査（全例調査）等における薬剤性肺障害の発現状況

薬剤名	調査症例数	薬剤性肺障害発現例数（%）	対象腫瘍
erlotinib	4,662例（調査票回収済み症例）	237（5.08）	肺癌
bortezomib	525例（調査票回収済み症例）	22（4.19）	多発性骨髄腫
cetuximab	3,436例（市販直後調査における推定使用患者数）	16（0.47）	大腸癌
sorafenib	2,010例（登録症例）	5（0.25）	腎細胞癌
bevacizumab	2698例（調査票回収済み症例）	10（0.37）	大腸癌
sunitinib	664例（市販直後調査における推定使用患者数）	2（0.30）	腎細胞癌

注：いずれも現時点では調査中の中間成績であり，発現頻度は調査終了後の解析により変化し得る．

sorafenibは，「根治切除不能または転移性の腎細胞癌」に対して2008年に承認され，次いで「根治切除不能または転移性の肝細胞癌」に対しても2009年に承認されたマルチキナーゼ阻害薬である．sorafenibでは，「ネクサバール錠 特定使用成績調査（全例調査）」が実施されており，2008年12月末時点においては，投与された2,010例中，5例のILDが報告され，そのうち2例が死亡に至っている（ネクサバール適正使用ガイド：肝細胞癌編，バイエル薬品）．sorafenibによるILDは全例が胸水を伴っており，特徴的な所見である可能性がある．

sunitinibは「イマチニブ抵抗性の消化管間質腫瘍ならびに根治切除不能または転移性の腎細胞癌」に対して2008年に承認されたマルチキナーゼ阻害薬である．「スーテントカプセル12.5 mg特定使用成績調査」が全例調査として実施されている．市販直後調査結果（推定使用患者数664例）では，2例のILDが報告され，そのうち1例が死亡に至っている（スーテント市販直後調査結果のお知らせ，ファイザー）．

最後に，全例調査が実施されている分子標的治療薬における薬剤性肺障

害の発現状況について表33にまとめた.

4　予防対策

まず重要なことは「EGFRチロシンキナーゼ阻害薬による間質性肺炎」のような重篤な有害事象は予防することが理想であり，投与に際してはリスクとベネフィットを十分に考慮した症例選択が重要である．今回erlotinibの肺障害の発現頻度および病態などがgefitinibと酷似していることが明らかになった．治療を選択する際，進められている危険因子の情報を十分に得ること，そして上記危険因子や効果予測の情報をしっかりと患者に理解してもらうことが重要である．

また，新しい薬剤については，治験段階で得られる日本人の安全性情報は限られており，さらに製造販売後においては多数の患者に使用されるだけではなく，組み入れ基準や除外基準で投与対象がある程度限定された治験実施時と異なり，幅広い投与対象となることも予測され，思わぬ安全性上の問題点が浮上してくる可能性も否定できない．したがって，新しい薬剤の製造販売後においては，日本人の安全性の確保を行うとともに，いかにして詳細かつ精度の高い安全性情報を蓄積するかという全体的な体制の問題も重要となる．薬剤性肺障害という有害事象には，人種差のある可能性は十分に想定されるが，その発現頻度は開発段階で明確に示し得るほど高くない場合が多い．現在，基本的になされている全例調査は当初，施設を限定し専門家の使用情報を得ることにより安全性を確保し，適正使用法を検討していく体制といえる．このような新しい薬剤を用いる場合，この仕組みを有効に利用し，常に最新の情報を得ることを習慣とすることが望まれる．

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Paracrine Factors of Multipotent Stromal Cells Ameliorate Lung Injury in an Elastase-induced Emphysema Model

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Multipotent stromal cells (MSCs) ameliorate several types of lung injury. The differentiation of MSCs into specific cells at the injury site has been considered as the important process in the MSC effect. However, although MSCs reduce destruction in an elastase-induced lung emphysema model, MSC differentiation is relatively rare, suggesting that MSC differentiation into specific cells does not adequately explain the recuperation observed. Humoral factors secreted by MSCs may also play an important role in ameliorating emphysema. To confirm this hypothesis, emphysema was induced in the lungs of C57BL/6 mice by intratracheal elastase injection 14 days before intratracheal MSC or phosphate-buffered saline (PBS) administration. Thereafter, lungs were collected at several time points and evaluated. Our results showed that MSCs reduced the destruction in elastase-induced emphysema. Furthermore, double immunofluorescence staining revealed infrequent MSC engraftment and differentiation into epithelial cells. Real-time PCR showed increased levels of hepatocyte growth factor (HGF) and epidermal growth factor (EGF). Real-time PCR and western blotting showed enhanced production of secretory leukocyte protease inhibitor (SLPI) in the lung. *In-vitro* coculture studies confirmed the *in vivo* observations. Our findings suggest that paracrine factors derived from MSCs is the main mechanism for the protection of lung tissues from elastase injury.

Received 15 January 2010; accepted 16 August 2010; published online 14 September 2010. doi:10.1038/mt.2010.192

INTRODUCTION

Lung emphysema is a smoking-related disease. Its pathological changes are defined as the permanent enlargement of airspaces distal to the terminal bronchioles, accompanied by destruction of their walls but without obvious fibrosis.[1] The course of this disease is irreversible, and there is currently no treatment that is effective in decreasing its destructive effects.[2] Consequently, innovative treatments should be investigated. Multipotent stromal cells (MSCs) have the capability of differentiating into various cell types, including endothelial cells, epithelial cells, adipocytes, and osteocytes.[3] Systemic administration of MSC is potentially simple and provides a direct therapeutic option for pulmonary diseases.[3] Many studies have shown the engraftment capabilities of MSCs in lungs[4–6] and the ability of these cells to differentiate into type I and type II epithelial cells, endothelial cells, and fibroblasts.[7] This engraftment can result from the systemic injection of allogeneic MSCs[4] or autologous MSCs.[8] Despite the low rates of engraftment,[9] the administration of these cells can decrease the intensity of damage caused to the lung by lipopolysaccharides,[6] elastase,[8] bleomycin,[10] naphthalene,[11] and asbestos.[12] These observations suggest that the mechanisms by which MSCs protect the lung might not only be via their ability to engraft and differentiate but also by other mechanisms. Previously, it has been shown that MSCs can release several types of cytokines, hormones, and growth factors to protect various tissues; these include hepatocyte growth factor (HGF),[13] epidermal growth factor (EGF),[14] and others.[15,16] In general, MSCs secrete soluble factors that are thought to play a significant role in tissue repair rather than transdifferentiation.[17] We hypothesized that the intratracheal administration of MSCs into lung tissue affected by elastase-induced emphysema would ameliorate the severity of the disease via their differentiation into various cells required for healing at the sites of injury. MSCs significantly reduced damage in the alveoli and were engrafted at injury sites; however, they disappeared from the lung tissue within a few weeks. Since, we found few differentiated MSCs in the lung tissues, we hypothesized that other factors, such as humoral factors, secreted by the MSCs may play an important role in ameliorating tissue damage caused by emphysema. We therefore evaluated the levels of HGF, EGF, secretory leukocyte protease inhibitor (SLPI), and interleukin-1β (IL-1β) in the lung tissues following MSC administration.

RESULTS

Phenotype and differentiation of MSCs

To confirm that the cells used in our experiments were enriched with MSCs, we examined the cell surface markers. Flow cytometric analysis showed that surface marker expression of these

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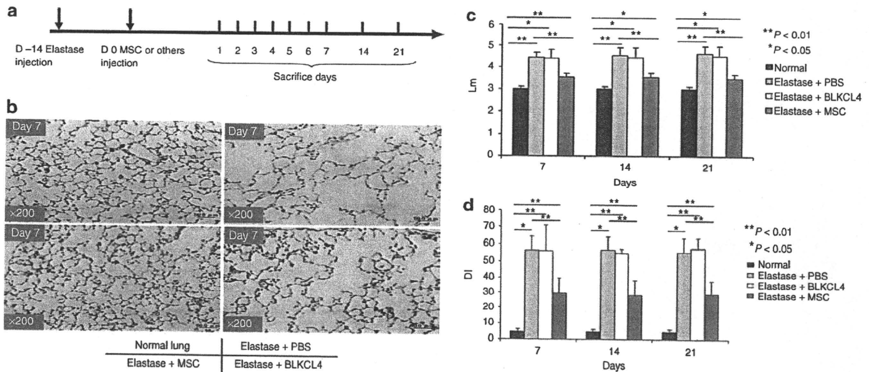

Figure 1 MSCs reduced elastase-induced emphysema. (**a**) The timeline of experiments. Mice were intratracheally injected with elastase 14 days before intratracheal injection with 5 × 10^5 MSCs, mouse fibroblasts (BLKCL4), or PBS. Mice were sacrificed at days 1, 2, 3, 4, 5, 6, 7, 14, and 21 as shown (n = ≥5/group). (**b**) Representative histological findings (hematoxylin and eosin staining). Treatment with MSCs reduced the elastase-induced emphysema, whereas BLKCL4 (fibroblast cell line) did not reduce these changes. Original magnification ×200. Bars = 50 μm. (**c**) The mean linear intercepts (Lm) for normal, elastase + PBS-, elastase + BLKCL4-, and elastase + MSC-treated groups. MSCs reduced the emphysematous changes; however, BLKCL4 did not. (**d**) MSCs restored the lung structure as assayed using the destructive index "DI" compared to normal, elastase + PBS-, and elastase + BLKCL4-treated groups. DI, destructive index; MSC, multipotent stromal cell; PBS, phosphate-buffered saline.

Table 1 A summary of the numbers of detected GFP$^+$ MSCs in lungs

Day	Lung injury	Engrafted cells, average per group (30 fields/mouse)	Differentiated cells, average per group (AQP5$^+$) (30 fields/mouse)
7	Yes	20	4
7	No	1	0
14	Yes	10	0
14	No	0	0
21	Yes	4	0
21	No	0	0

Abbreviations: AQP5, aquaporin 5; GFP, green fluorescent protein; MSC, multipotent stromal cell.
n = ≥5 mice/group.

cells was: CD105$^+$ (90.3%), CD73$^+$ (88.9%) CD90$^+$ (92.0%), CD45$^-$ (7%), and CD11b$^-$ (6%) (**Supplementary Figure S1a**). Furthermore, the MSCs used in the present study retained their differentiation capability. MSCs cultured at passage five readily differentiated into adipocytes when incubated in adipogenic maintenance medium and differentiated into osteoblasts following supplementation of the medium with osteogenic induction medium (**Supplementary Figure S1b**).

Intratracheal injection of MSCs reduced the development of elastase-induced emphysema

In order to understand the effect of injecting MSCs into lungs with elastase-induced emphysema, elastase was administrated into the trachea of mice. After 14 days, the mice were randomly assigned to receive intratracheal injections of MSCs, BLKCL4 (lung fibroblast cell line; negative control), or phosphate-buffered saline (PBS). For histological evaluation, mice were sacrificed at days 7, 14, and 21 (Figure 1a). Histological analysis showed that MSCs preserved the alveolar structure of mouse lungs, whereas BLKCL4 and PBS failed to preserve the structure (Figure 1b). Furthermore, the mean linear intercept "Lm" values[8,18] and destructive index[19] were measured to evaluate the severity of emphysematous changes. MSCs reduced the Lm; however, BLKCL4 failed to reduce the Lm compared with the elastase + PBS group (Figure 1c). Consistently, MSCs reduced the destructive index compared with the elastase + BLKCL4 group (Figure 1d). These results suggest that MSCs are able to ameliorate elastase-induced emphysematous changes.

GFP$^+$ MSCs engrafted in the injury sites but most MSCs did not differentiate into specific lung epithelial cells

To identify the possible mechanism by which MSCs could ameliorate the emphysematous changes, we performed immunohistochemical analysis for green fluorescent protein-positive (GFP$^+$) MSC cells following the same protocol as described above (Figure 1a). GFP$^+$ MSCs were detected after 7 days (Table 1 and Figure 2a). Furthermore, GFP$^+$ MSCs were detectable at days 14 and 21 but with decreased frequency (Table 1). To evaluate whether these MSCs had merely engrafted or had differentiated into epithelial cells, double immunofluorescence staining was performed. We presumed that the MSCs would differentiate into epithelial cells and accordingly screened for the presence of epithelial cell markers, such as alveolar epithelial type 1 marker aquaporin 5,[20] on these cells. Previous studies have shown that the contribution of adult bone marrow-derived cells in the restoration of cystic fibrosis-affected lung epithelium is low (0.025–0.01%).[21]

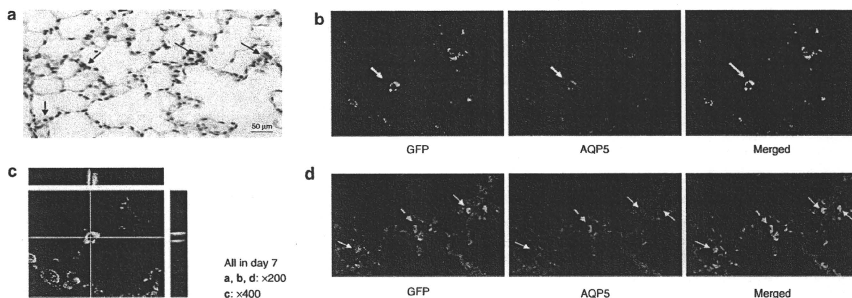

Figure 2 MSCs engrafted and differentiated into specific cells in the lung. **(a)** GFP+ MSCs engrafted in the lung. Arrows indicate GFP+ MSCs (shown at day 7; Original magnification ×200). **(b)** An MSC cell showed staining for two markers. Immunofluorescence staining at day 7 indicates a differentiated cell. Anti-GFP (green), anti-AQP5 (red), and merged (yellow). Original magnification ×200. **(c)** x–z confocal imaging of the cell shown in **c**. Original magnification ×400. **(d)** Immunofluorescence staining at day 7 shows that most MSCs had not differentiated because they continued to exhibit native green fluorescence only. White arrows show undifferentiated cells, blue dashed arrow show a differentiated cell. The proportions are shown in Table 1. Anti-GFP (green), anti-AQP5 (red), and merged (yellow) Original magnification ×200. The nuclei were stained with DAPI. AQP5, aquaporin 5; GFP, green fluorescent protein; MSC, multipotent stromal cell.

Our results showed that at day 7 only a small proportion of the injected cells positively expressed aquaporin 5 (Table 1 and Figure 2b). We confirmed the engraftment and differentiation of these cells by confocal microscopy (Figure 2c). We found no GFP+ MSCs that expressed another alveolar epithelial type 1 marker, podoplanin.[20] Neither could we detect the alveolar type 2 cell marker, pro-surfactant protein C (data not shown). Many of the GFP+ MSCs did not differentiate into specific lung cells because they did not show double-positive staining and only their native green fluorescence was expressed (Figure 2d). Notably, the numbers of GFP+-stained cells were also lower on days 14 and 21, similar to the results of immunohistochemical staining; we could find no double-positive cells after day 7 (Table 1). These data suggest that the ability of MSCs to engraft and differentiate does not adequately explain the amelioration of emphysematous injuries.

Proinflammatory cytokine IL-1β level was reduced in bronchoalveolar lavage fluid and at total lung mRNA levels in response to MSCs induction

In order to identify other possible mechanisms by which MSCs exert their effects, we first assayed the levels of the inflammatory cytokine IL-1β in the lung. IL-1β is responsible for distal airspace enlargement that is consistent with emphysema,[22] and it has been shown that the severity of elastase-induced emphysema is decreased in IL-1β knockout mice.[23] Recently, it was shown that the development of elastase-induced emphysema depends mainly on inflammasome activation by IL-1β via the IL-1R1/MyD88 signaling pathway.[24] Furthermore, it has been reported that MSCs inhibit the production of inflammatory cytokines such as IL-1β.[25] In our study, the levels of IL-1β in the bronchoalveolar lavage fluid decreased gradually after MSCs injection compared with levels in the nontreated groups (Figure 3a). The same pattern was noticed in the wild-type group that received PBS only without elastase. This was confirmed by real-time PCR for total lung mRNA, which showed significant

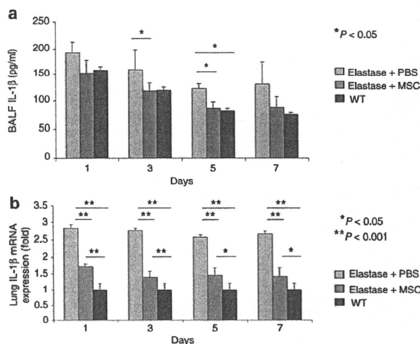

Figure 3 Inflammatory factor (IL-1β) was decreased in lungs treated by intratracheal administration of MSCs. **(a)** IL-1β concentration in BALF was measured by ELISA. IL-1β concentration was decreased to the normal levels of the WT groups compared to the elastase + PBS group. **(b)** Real-time PCR shows a significant decrease in mRNA levels of IL-1β in the MSC-treated groups. Representative data of the first week is shown. Levels of 1L-1β were not changed during the first week. AQP5, aquaporin 5; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; IL-1β, interleukin-1β; WT, wild-type.

differences in the IL-1β mRNA levels between the treated and both nontreated and wild-type groups (Figure 3b).

Injection of MSCs resulted in upregulation of growth factors and ameliorated alveolar damage in elastase-induced emphysema

To identify the possible factors produced or induced by the MSCs that affect emphysematous lungs, we assayed HGF levels. HGF

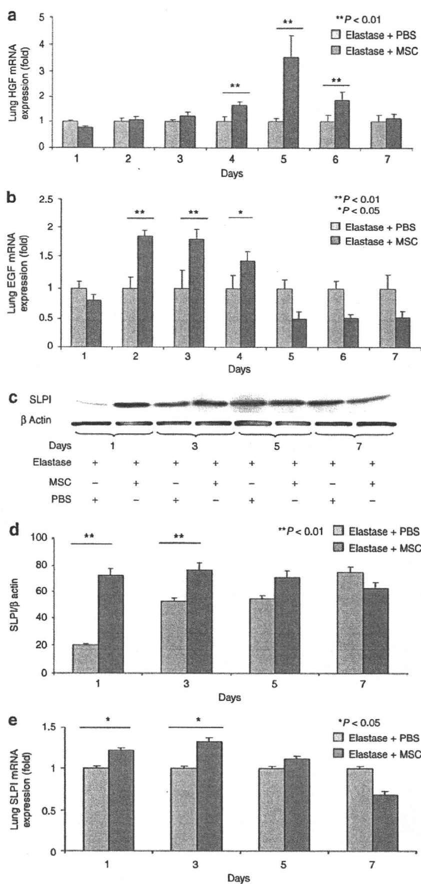

Figure 4 Growth factors (HGF, EGF) and protection factor for epithelial cells (SLPI; secretory leukocyte protein inhibitor) were increased in lungs treated by intratracheal administration of MSCs. **(a)** mRNA levels of HGF were increased in the MSC-treated group. **(b)** mRNA levels of EGF were increased in the MSC-treated group. **(c)** Western blotting of whole lung extracts shows increased expression of SLPI in the treated mice compared with the nontreated mice. **(d)** The SLPI bands were further analyzed by densitometry in comparison with the bands of β-actin. **(e)** mRNA levels of SLPI were significantly increased in the MSCs-treated groups at days 1 and 3 and slightly higher at day 5. Representative data of the first week is shown as levels of SLPI were not changed dramatically during the first 5 days; however, it was downregulated thereafter. EGF, epidermal growth factor; HGF, hepatocyte growth factor; MSC, multipotent stromal cell; PBS, phosphate-buffered saline.

is known for its effects in repairing the destruction occurring in emphysematous lungs induced by elastase injection.[26,27] Our analysis showed a gradual increase in HGF mRNA levels in MSC-treated lungs beginning from day 3 through day 4 peaked at day 5 and decreased thereafter (**Figure 4a**). Analyses for other growth factors also showed that EGF mRNA levels were significantly greater than baseline values starting from day 2 through day 3, but were decreased starting from day 4 (**Figure 4b**). EGF influences the proliferation of many structural cells, including fibroblasts and airway smooth muscle cells, as well as the turnover of matrix proteins.[28] EGF is also known for its effect on SLPI;[29] therefore, we examined the levels of SLPI protein in the lung. SLPI is responsible for protecting local tissues against inflammation and acts by inhibiting proteases such as elastase.[30] Furthermore, it has been suggested that SLPI can protect lungs against the development of emphysema even in α-1-antitrypsin-deficient individuals.[31] Our results showed that SLPI expression in treated lungs was significantly increased at both the protein and mRNA levels on days 1 and 3 (**Figure 4c–e**) in concert with EGF levels, but it was downregulated on days 5 and 7. Although EGF is known to induce SLPI in other systems, we found SLPI peaked at days 1 and 3 whereas EGF peaked at day 3. It is more likely that induction of SLPI is mediated by other factors.

Increased production of IL-1RA, HGF, and EGF by MSCs in coculture with the murine lung epithelial cell line MLE-12

In order to confirm the above results, we conducted experiments using a Transwell coculture system (**Figure 5a**). The murine lung epithelial MLE-12 cell line was used as an *in vitro* model, as described previously.[32] We confirmed that elastase at a concentration of 0.01 U/ml does not affect the viability of MSCs or MLE-12 cells as determined by measurement of lactate-dehydrogenase activity (LDH Cytotoxicity Assay; **Supplementary Figure S2a,b**). Furthermore, we confirmed that MSCs do not produce HGF or EGF when cultured with elastase (**Supplementary Figure S2c,d**). HGF and EGF mRNA levels in MSCs at 72 hours of coculture were significantly increased in the presence of MLE-12 cells with elastase (**Figure 5b,c**). To confirm that SLPI production was increased in concert with an increase in the levels of EGF, the complementary DNA of MLE-12 cells was amplified after 72 hours of coculture. SLPI mRNA of MLE-12 was notably higher in cells when elastase and MSCs were both present in the coculture system, whereas no increase in SLPI mRNA was observed in the presence of only elastase or MSCs in compare to coculturing well with elastase (**Figure 5d**). However, in both MSCs and MLE-12 cells, the levels of HGF, EGF, and SLPI were returned near to the baseline level after 120 hours of coculture (data not shown). MSCs have been reported to produce IL-1 receptor antagonist (IL-1RA),[33] and it was recently reported that IL-1RA diminishes acute elastase-induced emphysema by blocking IL-1.[24] We therefore examined IL-1RA levels after 72 and 120 hours (**Figure 5e**) and found that MSCs produced IL-1RA. We confirmed that such production was not caused by elastase (**Supplementary Figure S2e**). To confirm that MLE-12 produced IL-1β *in vitro*, we assayed the IL-1β levels in MLE-12 culture medium. Our data showed that MLE-12 produced IL-1β when elastase exists in the culture medium with or without

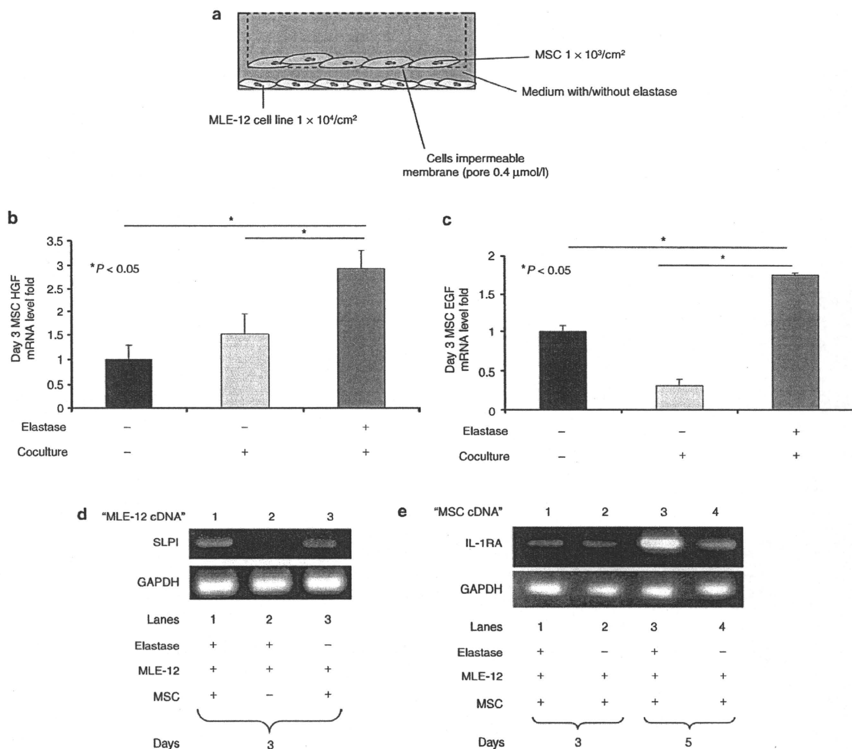

Figure 5 mRNA levels of HGF, EGF, and IL-1RA from MSCs were upregulated when cocultured with the mouse lung epithelial cell line MLE-12. **(a)** In the coculture experiments, MSCs were seeded at 1 × 10³/cm² in the upper chamber. MLE-12 cells were seeded at 1 × 10⁴/cm² in the lower chamber with or without 0.01 U/ml of elastase. Secreted proteins, but not cells, are able to pass through the membrane (pore size, 0.4 µm). **(b)** The HGF mRNA level of MSCs was increased in the coculture with MLE-12 cells. **(c)** The EGF mRNA level of MSCs was increased in the coculture with MLE-12 cells. **(d)** The SLPI mRNA level of MLE-12 cells was increased in the coculture with MSCs. **(e)** The IL-1RA mRNA level of MSC was increased in the coculture with MLE-12 cells. cDNA, complementary DNA; EGF, epidermal growth factor; HGF, hepatocyte growth factor; IL-1RA, IL-1 receptor antagonist; MSC, multipotent stromal cell; SLPI, secretory leukocyte protein inhibitor.

MSCs at days 3 and 5. However, IL-1β levels where undetectable in the absence of elastase with or without MSCs coculturing at days 3 and 5 (**Supplementary Figure S3**). This result suggests that MSCs can block the harmful action of IL-1β in our model.

Knocking down EGF in MSCs lowers SLPI production and recombinant EGF induces SLPI production in MLE-12 cells

In order to confirm that EGF from MSCs stimulates SLPI production in MLE-12 cells, we used the small interfering RNA (siRNA) knockdown technique to decrease EGF levels in MSCs. First, we examined whether siRNA could significantly affect EGF production at 72 hours compared with the production in negative controls (Figure 6a). Next, we used the coculture system described earlier (Figure 5a) with siRNA probes. Real-time analysis showed that SLPI production by MLE-12 cells decreased in the presence of elastase in the coculture system using MSCs with siRNA knockdown of EGF (Figure 6b). To further confirm this relationship, we investigated whether recombinant mouse EGF (rEGF) would enhance SLPI production when added to MLE-12 cells in culture. The results showed that SLPI mRNA levels were significantly increased at various concentrations of rEGF. SLPI mRNA levels were increased at 20 pg/ml

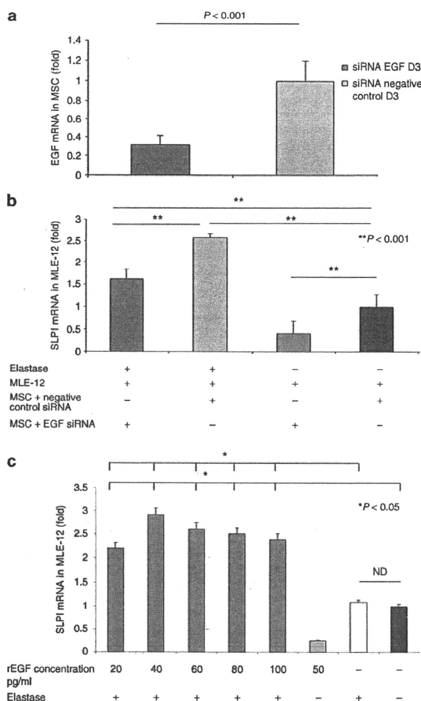

Figure 6 SLPI was decreased when EGF was knocked down in MSCs by siRNA. **(a)** siRNA knocked down EGF production in MSCs. **(b)** When MLE-12 cells were cocultured with MSCs transfected with siRNA against EGF or negative control, with or without elastase, the SLPI production of MLE-12 cells was decreased only in the case of MSCs transfected with siRNA against EGF and cultured with elastase. **(c)** Adding various concentrations of recombinant EGF (rEGF) to the culture medium containing elastase resulted in an increased production of SLPI in MLE-12 cells. Such increase was not noticed when rEGF or elastase alone were exist in the culture medium as assayed by real time PCR. EGF, epidermal growth factor; MSC, multipotent stromal cell; siRNA, small interfering RN; SLPI, secretory leukocyte protein inhibitor.

of rEGF, peaked at 40 pg/ml, and then progressively decreased at 60, 80, and 100 pg/ml (Figure 6c). However, elastase alone or rEGF presented alone in the medium at a concentration of 50 pg/ml did not enhance the production of SLPI (Figure 6c). These data confirm the novel effect of MSCs in inducing SLPI production through the release of EGF in the MLE-12 epithelial cell line.

DISCUSSION

Our findings suggest that MSCs ameliorate elastase-induced emphysematous damage through the release of soluble humoral factors rather than by engraftment in the lung tissue. Briefly, we consider that the effects of MSCs are exerted through the inhibition of IL-1β produced *in vivo* mainly by macrophage and by epithelial cells in our *in vitro* model. The upregulation of HGF and EGF in MSCs-treated animals promotes the repair of injuries, whereas the increase in SLPI production protects the lung tissue from the damaging effects of proteases. Our study showed that MSCs can ameliorate elastase-induced emphysema even when they are introduced after 14 days of injury; further, they can decrease the destruction of the alveolar structure in damaged lungs. Our study also showed that this effect of MSCs is mediated through paracrine mechanisms.

Elastase has been widely used as a pathogenic factor to induce emphysematous changes in lungs of several animals. Studies using this model are recent and repeatable.[8,24,26,32] Elastase causes massive degradation of the extracellular matrix and tissue cells, releasing a broad variety of degradation products and inflammatory mediators.[24] In our study, we detected MSC engraftment even at 3 weeks after administration, but found that only a few MSCs had differentiated into alveolar type 1 cells using aquaporin 5 as a marker. Furthermore, we found no cells positive for podoplanin (type 1) or pro-surfactant protein C (type 2) (data not shown). Although with the aid of confocal microscopy we were able to confirm that certain MSCs had differentiated into alveolar cells, it is unclear whether this alone would be sufficient to cause the observed effects. A number of studies have already reported that MSCs' effect is a paracrine-mediated fashion. This was the case in several types of organs' injuries like myocardial infarction,[16] bleomycin,[33] neonatal lung injury,[34,35] sepsis,[36] and endotoxin-induced lung injury.[37–39] Our study is the first report to show this mechanism to be the main mechanism in ameliorating elastase-induced emphysema. We observed increased levels of HGF, EGF, and SLPI in the treated animals in response to MSC injection, and the release of HGF and EGF from MSC in *in vitro* experiments. Furthermore, our study presents that SLPI production is induced in epithelial cells *in vitro* by EGF released from MSCs. The siRNA experiments and the administration of rEGF confirmed this *in vitro* novel relationship between SLPI and MSC-released EGF. Several reports showed MSCs' effect by assaying the levels of proinflammatory cytokines like "IL-1β, IL-6, tumor necrosis factor-α, interferon-γ" and anti-inflammatory cytokines such as "IL-1RA and IL-10."[33,34,37,40] Other studies focused on certain growth factor like keratinocyte growth factor[38] and only limited studies provided hypothesis of how a MSCs' product like prostaglandin E_2 will upregulate anti-inflammatory cytokine IL-10.[36] In our study, we are focusing on growth factors like HGF and EGF among other candidates rather than just the downregulation of IL-1β levels. Furthermore, our *in vitro* experiments showed that such factors might upregulate other factors like SLPI. SLPI protects the lung from the tissue degradation caused by proteases. However, the exact mechanism of this effect in lungs is poorly understood. It has been suggested that SLPI works in partnership with α-1-proteinase inhibitor (the major serum inhibitor of neutrophil elastase) to control the destructive activity of elastase.[41] Investigations of the role of SLPI in wound healing and lung cancer suggest that it targets various proteins, such as epithelin, tumor necrosis factor-α, monocyte chemoattractant protein-1, and IL-6.[42] This indicates the multiple effects and

complicated mechanism of SLPI in healing and protection against proteases. Considering that only a few cells engrafted and differentiated, this indicates that the production of such cytokines might not have been derived mainly from these cells; as such levels were not sustainable for >5 days after introducing MSCs. This could also explain the transient increase of these factors. In this context, it has been shown previously that there is similar variation in the levels of some anti-inflammatory cytokines after MSC injection at different time points.[39] Moreover, the time differential in the decreases of HGF and EGF levels suggest that the MSCs *in vivo* act as sub-populations that respond differently rather than as a homologous group of cells. One possible mechanism by which the MSCs mediate damaged tissue recovery is the "touch and go" mechanism,[43] *i.e.*, through rapid migration to the damaged organ and subsequent clearance following the release of stress-induced therapeutic molecules.[43] Our data support this mechanism and further suggest that the time necessary for this mechanism to operate is 3–5 days, which is possibly the life span of MSCs after injection. In general, the results of our study and other studies suggest two interesting points: (i) that there is a combination of factors[44] that involved in MSCs effect in each case which means a complex network of factors and cytokines integrated together at multilevels and (ii) although several reports showing the anti-inflammatory effect of MSCs is an IL-1RA- and IL-10-dependent effect, however, the other factors and cytokines are varying depending on the type of injury. In other words, there is no universal set of factors and cytokines to be released/induced by MSCs in all cases. Other studies used MSCs as vector for cell-based gene therapies showed that the efficiency of the treatment was enhanced in comparison to MSCs not transfected with any certain gene.[45,46] This further confirms that the paracrine mechanism is the main mechanism of MSCs. Further investigations are needed to elucidate the fate of the administered MSCs; whether they undergo apoptosis and/or acquire the properties of various cells was not considered in the present study. Furthermore, the long-term effects of the engrafted undifferentiated cells in lung tissue need to be clarified. Although our results suggest a clear mechanism for the observed effects of MSCs, it is possible that these effects occur via multiple mechanisms, including engraftment and differentiation.

In conclusion, we consider that MSCs protect lung tissues from injuries via paracrine mechanisms, including increased levels of HGF, EGF, and SLPI and decreased levels of IL-1β. Thus, the administration of MSCs may be a promising therapeutic approach for treating emphysema and other pulmonary diseases.

MATERIALS AND METHODS

Detailed descriptions of the following procedures can be found in the **Supplementary Materials and Methods**: mice and cell culture, the induction of adipogenic differentiation, histological analysis, protein and RNA isolation, real-time PCR and reverse transcription-PCR, bronchoalveolar lavage fluid collection and IL-1β measurement using ELISA, immunofluorescence and immunostaining, western blotting, and cytotoxicity assay.

Isolation of mouse MSCs and culture. The isolation of MSCs was performed as we reported previously.[47,48] Briefly, the bone marrow of the femurs of 6–8-week-old C57BL/6 or C57BL/6 GFP-transgenic mice (kindly provided by Dr M. Okabe, Osaka University, Osaka, Japan) was flushed out with culture medium. The cells were cultured in low-glucose Dulbecco's modified Eagle's medium (Gibco-BRL, Carlsbad, CA) containing 10% fetal bovine serum (certified for MSC culture; Gibco-BRL) along with 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco-BRL). After 72 hours, nonadherent cells were removed, and fresh medium was added. At 70–80% confluence, the cells were expanded and used after five passages.

The induction of emphysema in lung tissues and sample collection. Lung emphysema was induced by intratracheal injection of 0.01 (U/g body mass) porcine pancreas elastase (Sigma-Aldrich, St Louis, MO) as described previously[49] using a Microsprayer (Penn-Century, Philadelphia, PA). After 14 days, mice were randomly selected for intratracheal injection of MSCs, GFP^+ MSCs, BLKCL4, or PBS. The cells were injected at a concentration of 5×10^5 in 200 μl PBS. The groups used for histological analyses received MSCs, BLKCL4, or PBS, whereas those used for immunohistological and immunohistochemical analyses received MSCs, GFP^+ MSCs, or PBS. The mice were sacrificed at days 7, 14, and 21. To check the engraftment of MSCs in mice without lung injury, normal mice received an injection of GFP^+ MSCs (5×10^5 cells in 200 μl PBS). These mice were used a negative control group in immunohistological and immunohistochemical analyses. For molecular assays, mice in the MSC and PBS groups were sacrificed at days 1, 2, 3, 4, 5, 6, 7, 14, and 21 after MSC injection. Lung protein and RNA were extraction as described in the **Supplementary Materials and Methods.**

Coculture experiments. Cells of the MLE-12 lung epithelial cell line (1×10^4/cm^2) with or without elastase (0.01 U/ml) were cultured in the lower chambers of a 6-well Transwell system (Corning #2450, New York) with or without MSCs (1×10^3/cm^2), which were cultured in the upper chambers for 3 and 5 days. The membrane between the upper and lower chambers (pore size 0.4 μm) is impermeable to cells but permeable to humoral factors. The media were collected and cell RNAs were extracted. The samples were then assayed using real-time PCR and RT-PCR.

siRNA experiments. For silencing experiments, we used siRNA against EGF (Silencer Predesigned siRNA; Ambion, Austin, TX) and negative control (Silencer FAM-labeled negative control#1 siRNA; Ambion). siRNA transfection was carried out using a commercial kit (siPORT Neo FX; Ambion). The knockdown of EGF in MSCs was confirmed after 24, 48, and 72 hours by real-time PCR. Subsequently, MLE-12 and MSCs containing siRNA were cultured in Transwell plates as described above. The SLPI and EGF production of these cells was assayed at 72 hours after the start of coculture using real-time PCR. The following probe was used to knockdown the EGF: sense, CGGGAAGCAUCAUCGAAUAtt; antisense, UAUUCGAUGAUGCUUCCCGct.

Statistical analysis. All values are expressed as the mean ± SD. Differences between the groups were evaluated using a two-tailed unpaired Student's *t*-test with Sigmaplot version 11 (Hulinks, Tokyo, Japan). $P < 0.05$ was considered significant.

SUPPLEMENTARY MATERIAL

Figure S1. Phenotype and differentiation of MSCs.
Figure S2. Elastase showed no effect on viability or inducing cytokine production on MSCs and MLE-12.
Figure S3. IL-1β levels in MLE-12 culture medium.
Materials and Methods.

ACKNOWLEDGMENTS

This work was supported by a grant (050884) to A.M.K. and grants-in-aid for Scientific research (16390232) to Y.S. (19590878) to H.X., and (21590980) to S.O. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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ORIGINAL ARTICLE

Gefitinib or Chemotherapy for Non–Small-Cell Lung Cancer with Mutated EGFR

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ABSTRACT

BACKGROUND

Non–small-cell lung cancer with sensitive mutations of the epidermal growth factor receptor (EGFR) is highly responsive to EGFR tyrosine kinase inhibitors such as gefitinib, but little is known about how its efficacy and safety profile compares with that of standard chemotherapy.

METHODS

We randomly assigned 230 patients with metastatic, non–small-cell lung cancer and EGFR mutations who had not previously received chemotherapy to receive gefitinib or carboplatin–paclitaxel. The primary end point was progression-free survival; secondary end points included overall survival, response rate, and toxic effects.

RESULTS

In the planned interim analysis of data for the first 200 patients, progression-free survival was significantly longer in the gefitinib group than in the standard-chemotherapy group (hazard ratio for death or disease progression with gefitinib, 0.36; P<0.001), resulting in early termination of the study. The gefitinib group had a significantly longer median progression-free survival (10.8 months, vs. 5.4 months in the chemotherapy group; hazard ratio, 0.30; 95% confidence interval, 0.22 to 0.41; P<0.001), as well as a higher response rate (73.7% vs. 30.7%, P<0.001). The median overall survival was 30.5 months in the gefitinib group and 23.6 months in the chemotherapy group (P=0.31). The most common adverse events in the gefitinib group were rash (71.1%) and elevated aminotransferase levels (55.3%), and in the chemotherapy group, neutropenia (77.0%), anemia (64.6%), appetite loss (56.6%), and sensory neuropathy (54.9%). One patient receiving gefitinib died from interstitial lung disease.

CONCLUSIONS

First-line gefitinib for patients with advanced non–small-cell lung cancer who were selected on the basis of EGFR mutations improved progression-free survival, with acceptable toxicity, as compared with standard chemotherapy. (UMIN-CTR number, C000000376.)

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N Engl J Med 2010;362:2380-8.
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Non–small-cell lung cancer is a major cause of death from cancer. The use of cytotoxic chemotherapy is associated with a response rate of 20 to 35% and a median survival time of 10 to 12 months among patients with advanced non–small-cell lung cancer.[1,2] Gefitinib is an orally administered tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR). In two phase 2 studies of patients with previously treated non–small-cell lung cancer, the response rate was 9 to 19%.[3,4] In subsequent phase 3 trials, the noninferiority of gefitinib as compared with docetaxel with respect to overall survival was shown in one study (hazard ratio, 1.02)[5] but not another (hazard ratio, 1.12).[6] Meanwhile, demographic and clinical factors such as Asian race, female sex, nonsmoking status, and adenocarcinoma were shown to be predictive of the efficacy of gefitinib, warranting a large comparative trial (First Line Iressa vs. Carboplatin/Paclitaxel in Asia [IPASS]; ClinicalTrials.gov number, NCT00322452) in which patients were selected in accordance with these factors.[7]

In May 2004, two pivotal studies showed that the presence of somatic mutations in the kinase domain of EGFR strongly correlates with increased responsiveness to EGFR tyrosine kinase inhibitors in patients with non–small-cell lung cancer.[8,9] It was later found that subgroups of patients with non–small-cell lung cancer who had sensitivity to gefitinib had a high incidence of EGFR mutations. In Japan, 30% or more of patients with mutated-EGFR non–small-cell lung cancer are male or have a history of smoking.[10,11] Therefore, we hypothesized that selecting patients on the basis of EGFR mutations rather than clinical factors would result in a population with a greater sensitivity to gefitinib.

Our previous prospective, phase 2 studies of gefitinib therapy in patients with advanced non–small-cell lung cancer and EGFR mutations[12-14] revealed a response rate of more than 70% and progression-free survival of 9 to 10 months. We also developed a rapid, sensitive method for detecting sensitive EGFR mutations: the peptide nucleic acid–locked nucleic acid (PNA-LNA) polymerase-chain-reaction (PCR) clamp method.[15] We then undertook a phase 3 study comparing gefitinib and standard carboplatin–paclitaxel chemotherapy in patients who had advanced non–small-cell lung cancer with sensitive EGFR mutations and who had not previously received chemotherapy.

METHODS

PATIENT POPULATION

This multicenter, randomized, phase 3 trial was approved by the institutional review board of each participating center. Eligibility criteria included the presence of advanced non–small-cell lung cancer harboring sensitive EGFR mutations, the absence of the resistant EGFR mutation T790M (in which threonine at amino acid 790 is substituted by methionine), no history of chemotherapy, and an age of 75 years or younger (because a benefit of a platinum-based regimen in patients >75 years of age is not established). Table 1 in the Supplementary Appendix (available with the full text of this article at NEJM.org) lists the detailed eligibility and exclusion criteria. The authors attest to the fidelity of the article to the full protocol and statistical-analysis plan.

DETECTION OF *EGFR* MUTATIONS

Cytologic or histologic specimens were examined for *EGFR* mutations by means of the PNA-LNA PCR clamp method. Briefly, genomic DNA fragments containing mutation hot spots of the *EGFR* gene were amplified with the use of a PCR assay in the presence of a peptide nucleic acid clamp primer synthesized from a peptide nucleic acid with a wild-type sequence. This method results in preferential amplification of the mutant sequence, which is then detected by a fluorescent primer that incorporates locked nucleic acids to increase the specificity. As a result, a mutant *EGFR* sequence is detected in specimens that contain 100 to 1000 excess copies of wild-type *EGFR* sequence. The sensitivity and specificity of the PNA-LNA PCR clamp method are 97% and 100%, respectively.[15,16]

STUDY DESIGN AND TREATMENT

Before randomization, patients were stratified according to sex, clinical stage of non–small-cell lung cancer (IIIB, IV, or postoperative relapse), and institution. Eligible patients were randomly assigned to receive either gefitinib (at a dose of 250 mg per day orally) or standard chemotherapy. The standard chemotherapy consisted of paclitaxel (at a dose of 200 mg per square meter of body-surface area, given intravenously over a 3-hour period) and carboplatin (at a dose equivalent to an area under the concentration–time curve [AUC] of 6, given intravenously over a 1-hour period), both administered on the first day of every 3-week cycle. The

carboplatin dose in milligrams was calculated by means of the Calvert formula (AUC × [the calculated creatinine clearance in milliliters per minute + 25]; www.freekinetics.com/auccalc1.htm). The glomerular filtration rate was estimated according to the Cockcroft–Gault method ([140 − age in years] × [actual weight in kilograms] ÷ [72 × serum creatinine level in milligrams per deciliter {× 0.85 in women}]). Chemotherapy was continued for at least three cycles. Gefitinib was administered until disease progression, development of intolerable toxic effects, or withdrawal of consent.

CLINICAL ASSESSMENTS

Assessments made before enrollment are summarized in Table 2 in the Supplementary Appendix. Assessment of the tumor for a response to treatment was performed by means of computed tomography (CT) every 2 months. Unidirectional measurements were adopted on the basis of the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.0).[17] Progression-free survival was evaluated for the period from the date of randomization to the date when disease progression was first observed or death occurred. Treatment response and progression-free survival were determined by external review of the CT films by experts who were not aware of the treatment assignments. Overall survival was evaluated for the period from the date of randomization to the date of death. Toxic effects were assessed according to the National Cancer Institute Common Terminology Criteria (NCI-CTC, version 3.0; http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf).

STATISTICAL ANALYSIS

The primary end point was progression-free survival, as a measure of the superiority of gefitinib over carboplatin–paclitaxel. From our previous data, we hypothesized that the progression-free survival with gefitinib was 9.7 months; from the results of the Iressa NSCLC Trial Assessing Combination Treatment (INTACT),[18] we hypothesized that the progression-free survival with standard chemotherapy was 6.7 months. We estimated that a total of 230 events would be needed for the study to have a power of 80% to confirm the superiority of gefitinib over standard chemotherapy, with the use of a log-rank test and a two-sided significance level of 5%. Setting the duration of enrollment to 2 years with a minimum follow-up period of 6 months, we initially planned to enroll 320 patients.

Kaplan–Meier survival curves were drawn for progression-free survival and were compared by means of a log-rank test. Hazard ratios (and 95% confidence intervals) were calculated with the use of a Cox proportional-hazards analysis. Prespecified adjustment factors included sex and clinical stage.

Secondary end points included overall survival, response rate, time to the deterioration of performance status (Eastern Cooperative Oncology Group [ECOG] performance status score of ≥3, capability of only limited self-care, or confinement to a bed or chair for >50% of waking hours[19]), and toxic effects. Overall survival and the time to ECOG performance status score of 3 or more were analyzed in the same way as progression-free survival. The response rate and rate of toxic effects were compared between the two groups with Fisher's exact test and the Wilcoxon test, respectively. Each analysis was performed with the use of a two-sided, 5% significance level and a 95% confidence interval by means of SAS for Windows software (release 9.1, SAS Institute).

One interim analysis was planned to analyze the primary end point (significance level, $P=0.003$). The Lan–DeMets method was used to adjust for multiple comparisons. The O'Brien–Fleming type alpha-spending function was also used.

RESULTS

PATIENT CHARACTERISTICS

The study was begun in March 2006. The preplanned interim analysis was performed 4 months after the 200th patient was enrolled (May 2009); it showed a significant difference in progression-free survival between the two treatment groups ($P<0.001$), and the independent data and safety monitoring committee recommended termination of the study. Therefore, the study was stopped at the end of May 2009.[20]

In total, 230 patients were enrolled from 43 institutions in Japan (Fig. 1). Half (115 patients) were randomly assigned to receive gefitinib and half to receive carboplatin–paclitaxel. Two patients were excluded because they were found to be ineligible. In the chemotherapy group, 1 patient was not evaluated for safety, owing to lack of receipt of the study drugs, and 3 others were excluded from the analysis of progression-free survival.