表4　結核菌の生物学的特徴や病原性（小林，2005.[7]）

細胞内寄生性	桿菌（0.2-0.6 × 1-10μm），宿主細胞，特に，マクロファージ内で抗菌機構から逃れて増殖
細胞壁	脂質成分が豊富なため，疎水性であり，化学物質にも安定，グラム染色に難染色性，抗酸性
好気性	酸素分圧の高い臓器（肺など）で増殖し，病変を形成
遅発育性	至適温度：37℃，倍加時間：約12～15時間，培養集落形成に4～8週間
感染形式	飛沫核・空気感染
病原性	慢性炎症，肉芽腫，乾酪壊死，空洞形成，線維化
遺伝子	全ゲノム（約4.41 Mb）の解読

であるが，細胞壁が長鎖脂肪酸（ミコール酸）に富み，グラム染色では難染色性を示す．そのため，抗酸性（Ziehl-Neelsen，Kinyoun）染色や蛍光染色が用いられる．抗酸菌は“赤い桿菌”として観察される．抗酸菌以外の通常細菌やヒト組織・細胞は後染色のメチレンブルーにより“青く”対比染色される．抗酸菌をオーラミンやローダミンなどの蛍光色素を用いて染色し，「暗い背景」下に抗酸菌は「緑青–橙–黄色」の蛍光を発する．分裂倍加時間は約12～15時間の遅発育菌であり，感染伝播は飛沫核（空気）感染による．宿主防御機構では，マクロファージ–サイトカイン–T細胞応答系，すなわち，細胞性免疫が役割を演じ，細胞内殺菌物質としてガス状物質（反応性酸素化合物質や反応性窒素化合物質）が寄与している．その結果，結核菌感染者の約10％が一生涯において結核を発病する．病変は慢性炎症，肉芽腫，乾酪壊死，空洞形成や線維化などが特徴的である．*M. tuberculosis* H37Rvの全ゲノム塩基配列が解明された．今後，遺伝子解析を基盤とした科学的戦略が推進され，分子・遺伝子標的を視点とした新規診断法，抗結核薬の開発，薬剤耐性獲得機構の解明や新規ワクチン開発が展開されるであろう．

3. 結核の診断

結核は肺結核と肺外結核に分類されるが，85％以上は肺結核である．肺結核の症状として，持続性のものや2週間以上続く咳嗽や喀痰，血痰，胸痛，軽度発熱，体重減少がある．特に，持続性咳嗽と喀痰は重要である．肺外結核部位として，リンパ節，胸膜，泌尿生殖器，骨・関節，髄膜・中枢神経系，腹膜・消化管や心外膜などがある．

表5[7]に示したように診断には，病原体診断と補助診断がある．病原体診断は確定的であるが，図1[7]に示す塗抹検査陽性の場合，結核菌のみならず，非結核性抗酸菌を考慮する必要がある．現在，もっとも信頼性の高い検査は培養法であるが，欠点として長期間を要することである．固形培地であれば4～8週間，液体培地であれば10～14日間を要する．PCRなど遺伝子診断は迅速性，感度や特異性に優れるが，生死菌の識別や技術的問題（熟練，偽陽性・偽陰性）がある．

胸部X線所見では，浸潤影，結節，空洞，線維化，肺門リンパ節腫大や石灰化，無気肺，胸膜肥厚・癒着，胸水貯留など，多彩である（図1–d）．好発部位は肺尖を含む上肺や中肺野である．多発性びまん性結節陰影は播種（粟粒）性結核でみられる．これらの所見は他の炎症性や腫瘍性肺疾患にも認められる所見であり，結核に特異的でなく，注意を要する．あくまでも結核の補助的診断法である．

ツベルクリン皮内反応の陽性（日本の紅斑：直径10 mm以上，欧米の硬結：直径5 mm以上）は結核菌感染のみならず，BCG（Bacille Calmette-Guérin：弱毒ウシ型結核菌）接種や非結核性抗酸菌感染でもみられ，逆に，活動性結核の約25％は陰性である．陰性は真の陰性（結核菌未感染）や偽陰性（結核菌既感染にもかかわらず陰性）を包含し，偽陰性として，栄養障害，高齢者，免疫疾患，リンパ系悪性腫瘍，副腎皮質ステロイド薬療法，慢性腎不全，サルコイドーシス，AIDSを含むHIV感染者や重症結核（播種性）などがある．したがって，ツベルクリン皮内反応

表5　結核の診断（小林，2005，[7]）

病原体診断	塗抹検査	抗酸菌染色（Ziehl-Neelsen，Kinyoun染色），蛍光染色
	培養検査	固形培地（卵培地：小川，Löwenstein-Jensen， 寒天培地：Middlebrook 7H10，7H11）：4～8週間 液体培地（MGIT，MB check）：10～14日 薬剤感受性試験
	遺伝子検出	核酸増幅法：Polymerase Chain Reaction（PCR） DNA-DNAハイブリゼーション 薬剤耐性遺伝子
補助診断	胸部X線	中および上肺野病変（浸潤，結節や空洞） リンパ節腫大や石灰化 胸膜炎・胸水貯留
	病理学的検査	乾酪壊死を伴う肉芽腫
	TST	ツベルクリン皮内反応（Mantoux） 48時間後判定：遅延型皮内反応（IV型） 陽性：結核菌感染，BCG陽転，非結核性抗酸菌感染 陰性：未感染，BCG未接種，免疫不全（HIV／AIDS，重症結核，薬物性）
	IGRA：QFT	末梢血細胞IFN-γ産生・遊離試験（IGRA，Quantiferon）：*in vitro* 指標：IFN-γ 抗原：ツベルクリン蛋白質，RD1（ESAT-6，CFP-10）

は結核の補助診断である．ツベルクリン皮内反応陽性は感染防御の指標とならないことも留意する．

近年，結核菌特異的蛋白質抗原（ESAT-6やCFP-10）を用いた免疫学的診断法が開発され，臨床応用されている（interferon-γ遊離試験，Quantiferon）．これらの抗原はBCGや多くの非結核性抗酸菌に存在しないため，結核菌感染を特異的に検出できる．原理は，末梢血に特異的蛋白質抗原を加え，培養後，産生・遊離されるIFN-γを定量する（陽性：0.35　IU/mL以上）．検査対象として，①潜在性結核菌感染や②活動性結核の補助診断に応用されている．

4. 治　療

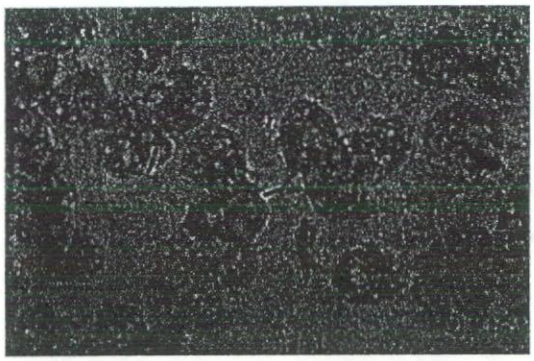
治療の原則は多剤併用抗結核化学療法である．結核菌の薬剤耐性は，各抗結核薬の標的に関与した遺伝子の変異により獲得され，多剤耐性はこれらの遺伝子の変異が集積することにより出現する．抗結核薬により，耐性菌出現頻度は異なるが，1薬剤あたり106～109例中1例であるため，薬剤を併用することにより，耐性菌の出現頻度を低下させることが可能となる．ただし，この場合，確実な服用は絶対条件である．そのため，WHOはDOTSを推進しており，標準的な治療で，服薬期間は約6カ月である．服用の組み合わせでは，最初の2カ月は4剤（INH+RIF+EMB+PZA），その後4カ月は2剤（INH+RIF）を用いる[2]．

薬剤耐性結核の原因は不適切な結核医療，すなわち，抗結核化学療法薬の不適切な選択や使用，治療中断や脱落であり，医療関係者や患者の対応に起因するman-made diseaseである．全世界で約5,000万人以上が多剤耐性結核菌（INHとRIFに同時耐性，MDR-TB）に既感染し，各国の医療費は薬剤感受性結核に比べ3～100倍を要し，再発率が28％と極めて高く，結核制圧対策の大きな課題である．加えて，超多剤耐性結核菌（XDR-TB）の出現は抗結核化学療法を困難にしている．

5. 予　防

予防は，感染源対策として患者の早期発見，治

(a)

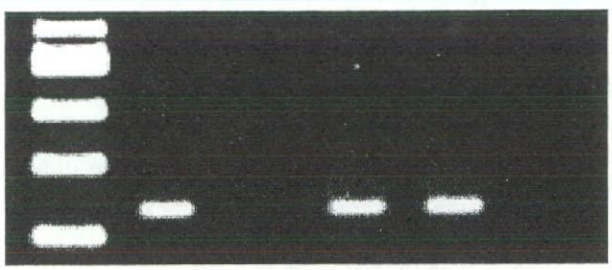


結核菌の喀痰塗抹検査（Ziehl-Neelsen 染色）．結核菌は赤染されている桿菌であり，ヒト組織は青染されている．

(b)

結核菌の培養所見．喀痰を卵（小川）培地に接種し，6週間後に多数の集落（乳白色–薄黄色）形成を認めた．

(c)



核酸増幅法による結核菌遺伝子の検出．結核菌 DNA に特異的なプライマーを用い，ポリメラーゼ連鎖反応で喀痰の結核菌遺伝子を検出した．分子サイズマーカー：レーン1，陽性：レーン2，4，5，陰性：レーン3，6

(d)

肺結核の胸部X線所見．浸潤影，結節，線維化，肺門リンパ節腫大，胸膜肥厚・癒着や胸水貯留など，多彩な所見を認めた．

図1　結核の診断（小林，2005,[7]）

療，接触者（家族，学校，会社など）の調査，さらに，予防接種や化学予防がある．予防接種はBCGが汎用されている．現行の結核発病予防ワクチンであるBCGの有効性に関し，根拠にもとづく医療（Evidence-Based Medicine: EBM）の観点から，見直しが進められた．その結果，乳幼児結核（結核性髄膜炎など播種性結核）への有効性は認められたが，成人肺結核に対するBCGの有効性は実証されないこと，さらに，BCG再接種によるツベルクリン皮内反応の陽転化が結核菌感染の診断の妨げとなること等の事由により，BCGは乳幼児期（原則として，生後6カ月までにツベルクリン皮内反応を省略したBCG直接接種）の初回接種のみに限定し，BCG再接種およびツベルクリン皮内反応（小学1年および中学1年時）は2003年4月から廃止された[8]．

化学予防としてはイソニアジド等の抗結核化学療法薬を服用し，結核の発症を防止する（効果は70～80%）．ただし，感染結核菌が化学療法薬に感受性であることが不可欠である．

6. 集団感染や施設内（院内）感染対策

結核の主要な感染源は排菌量の多い喀痰塗抹陽

表6　施設内結核感染防止対策（CDC, 2007,[9]）

管理対策	・施設内感染防止委員会や感染制御部隊（infection control team）の設置 ・院内結核発生動向調査 ・医療従事者へ結核に関する啓発 ・医療従事者のツベルクリン皮内反応 ・医療従事者のinterferon-γ遊離試験 ・持続性（2週間以上）咳嗽患者の優先診療 ・喀痰塗抹陽性結核患者の早期発見や迅速な個室収容・隔離，有効な抗結核化学療法
環境や設備整備計画	・陰圧個室の整備 ・換気（7回以上／時間） ・紫外線照射やHEPAフィルター装備
個人防御対策	・飛沫核を除去できるマスク（例：N95） ・咳嗽を誘発する医療行為（例：気管支内視鏡検査や気管内挿管）における細心の注意 ・未発病感染者の化学予防（INH服用など）

性肺結核や喉頭結核患者（塗抹陰性や肺外結核の感染性は低い）である．結核菌の感染経路は飛沫核（空気）感染様式であり，結核菌が直径1～5μmの落下し難い，浮遊性飛沫核に含まれ，吸入することで感染する．感染は結核菌の被曝露（吸入）者の約30%に成立する．

結核のみならず感染症の蔓延防止は感染源，感染経路および感受性宿主対策を基本としている．施設内結核感染防止対策は，①管理対策，②環境や設備整備計画，③個人防御対策から構成される（表6）[9]．

7. 結核予防法の統廃合

2007年4月の改正感染症法の施行に伴い，結核予防法は「感染症の予防および感染症の患者に対する医療に関する法律（感染症法）」に統廃合された[8]．結核は二類感染症に位置付けられ，結核を診断した場合，医師は直ちに最寄りの保健所長を経由し都道府県知事に届け出なければならない．また，生物テロ対策として，2007年6月から，「特定病原体等（一～四種）の管理規制」が施行されている．結核菌は空気感染病原体で，かつ個体に対して高い危険度を示すため，Biosafety level-3（BSL-3）など，施設内での取り扱いや保管の基準が定められた．多剤耐性結核菌は三種病原体であり，施設内での取り扱いや保管の基準に加え，所持に際し，厚生労働大臣へ届け出，また，運搬に際し，都道府県公安委員会へ届け出が必要である．結核菌（多剤耐性結核菌を除く）は四種病原体であり，施設内での取り扱いや保管の基準の遵守が必要である．

おわりに

結核は代表的な再興感染症であり，現在でも，人類に甚大な健康被害を提供している．結核対策には多くの課題が山積しているが，科学的根拠にもとづいた「感染源，感染経路，感受性宿主」対策や「診断，治療，予防」が実施され，結核が制圧されることを期待している．

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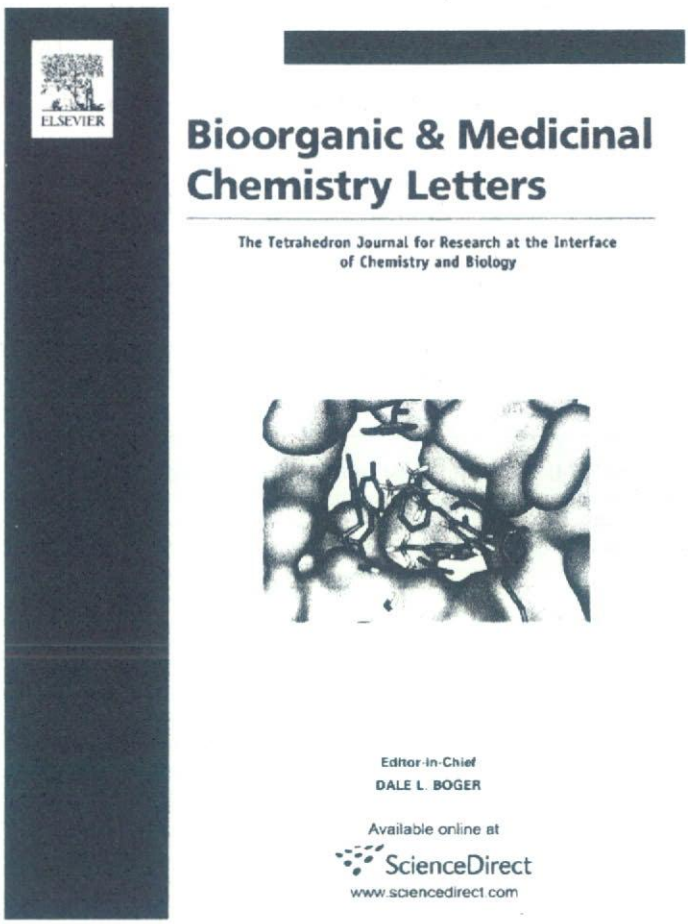

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Synthesis of new sugar derivatives from *Stachys sieboldi* Miq and antibacterial evaluation against *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Staphylococcus aureus*

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Abstract—A series of sugar derivatives (**7**–**14**) were synthesized from stachyose, a sugar compound of *Stachys sieboldi* Miq, and evaluated for antibacterial activity against *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Staphylococcus aureus*, and their structure–activity relationships were studied. The results showed that the compound OCT359 (allyl *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1 → 6)-*O*-(2,3,4-tri-*O*-acetyl-α-D-galactopyranosyl)-(1 → 6)-*O*-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside) (**12**) exhibited in vitro antibacterial activity. The allyl group at C-1 and the acetoxy groups of the manninotrioside were requisite for the antibacterial activity.

Manninotriose is obtained by hydrolysis of stachyose, a sugar compound abundantly present in the root of *Stachys sieboldi* Miq. Chung et al. have previously reported the synthesis of methyl *O*-β-D-galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 6)-*O*-β-D-glucopyranoside,[1] from manninotriose (**1**). Biological activities of manninotriose are largely unknown. Kubo et al. studied the biological effect of a stachyose-containing extract from the root of *Rehmannia glutinosa* on hemorheology. A 50% ethanolic extract from the steamed root of *R. glutinosa* decreased erythrocyte deformability, inhibited polybrene-induced erythrocyte aggregation, and promoted the activity of the fibrinolytic system.[2] In the present study, we showed that a new sugar compound derived from manninotriose exhibited antibacterial activity against *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Staphylococcus aureus*.

The trisaccharide OCT359 (allyl *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1 → 6)-*O*-(2,3,4-tri-*O*-acetyl-α-D-galactopyranosyl)-(1 → 6)-*O*-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside) (**12**) consists of one glucose and two galactose residues. This compound was prepared from manninotriose obtained by partial hydrolysis of stachyose (**1**), followed by acetylation, bromo-substitution, and allylation (Schemes 1 and 2). An aqueous solution of *O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 6)-*O*-D-glucopyranose (manninotriose), prepared from *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1 → 6)-*O*-(2,3,4-tri-*O*-acetyl-α-D-galacto-pyr (1,2,2′,2″, 3,3′,3″,4,4′,4″,6″-undeca-*O*-acetyl- β-manninotriose) (**2**) known in the literature and positive for Fehling's reaction, was treated with sodium borohydride to afford a sugar alcohol as an amorphous powder (94.6%) which was then negative for Fehling's reaction. After acetylation with acetic anhydride and pyridine, the acetate of a sugar alcohol was obtained as an amorphous powder (100%) and its ^{1}H NMR spectrum showed signals of acetyl groups at δ 1.96 (×2), 2.04, 2.05, 2.07, 2.08, 2.09, 2.13 (×2), 2.14 (×2), and 2.15 (Scheme 1).

The allyl group of OCT359 (**12**) was replaced with seven other types of functional groups; namely, methyl (**7**), ethyl (**8**), *n*-propyl (**9**), *i*-propyl (**10**), *n*-butyl (**11**), crotyl (**14**), and propargyl (**13**) (Scheme 2). The general method for modification is as follows. The mixture of *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1 → 6)-

Keywords: Stachyose; β-Allyl manninotrioside; Antibacterial activity.
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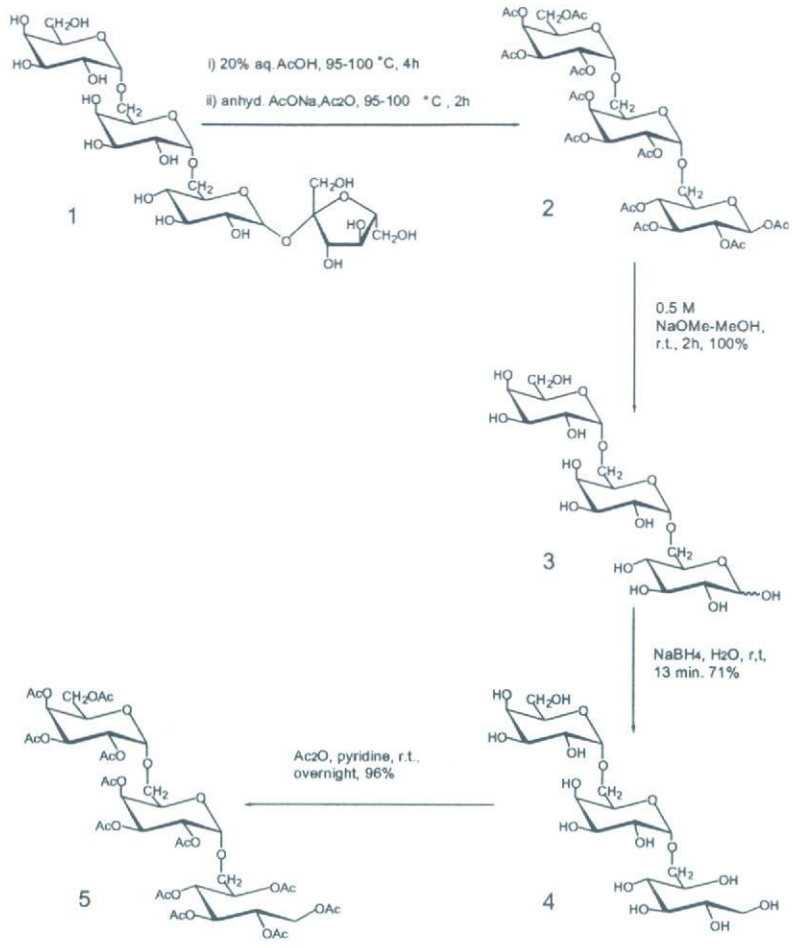
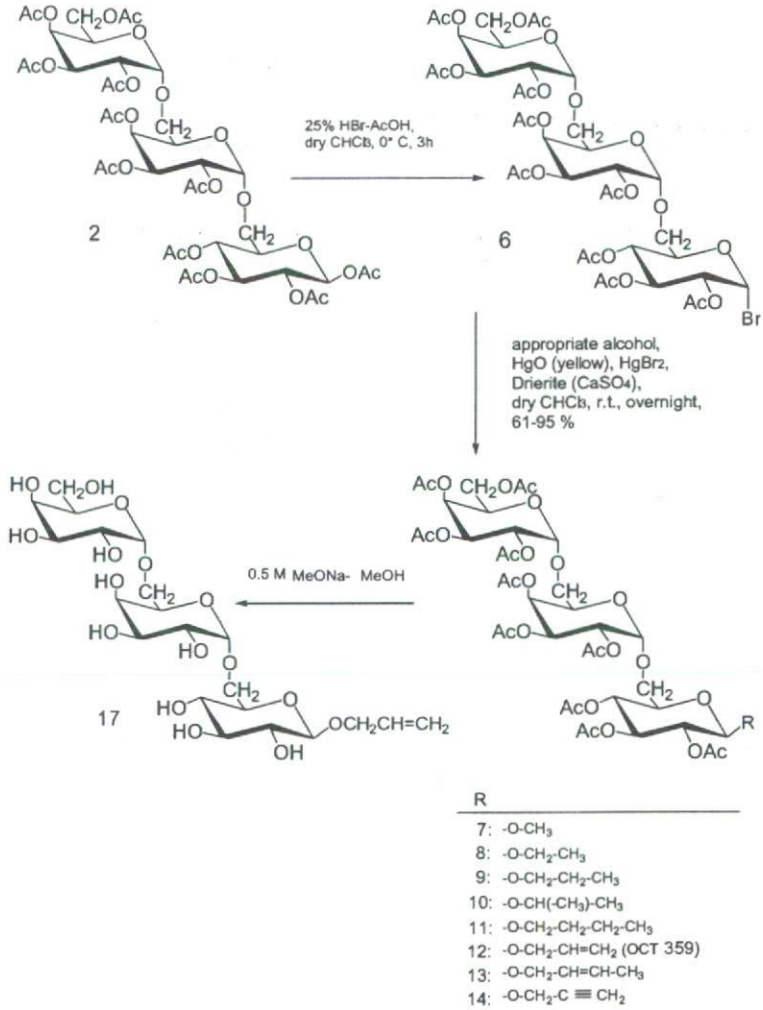


Scheme 1. Synthesis of sugar alcohol of manninotriose.

O-(2,3,4-tri-*O*-acetyl-α-D-galactopyranosyl)-(1 → 6)-*O*-2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide (2,2′,2″,3,3′,3″,4,4′,4″,6″-deca-*O*-acetyl-α-manninotriosyl bromide) (**6**), an appropriate alcohol, mercury (II) oxide (yellow), mercury (II) bromide, and Drierite (calcium sulfate) in dry chloroform was stirred overnight at room temperature. After workup, the resulting syrup or amorphous powder was purified by column chromatography. The yield of each compound was from ca. 61% to 94% (Scheme 2). The antibacterial activity of compounds (**7–14**) was investigated. The target bacteria were *M. tuberculosis* $H_{37}Rv$, *M. avium* 724S, and *S. aureus* including multi drug-resistant *M. tuberculosis* (MDR-MTB) and methicillin-resistant *S. aureus* MRSA) (Tables 1 and 2). The MICs of OCT359 (R=allyl) (**12**) with these bacteria were between 3.13 and 25 μg/ml. However, no other derivatives exhibit antibacterial activity (>400 μg/ml) (Table 1). The isomer (**16**) of α-configuration did not exhibit antibacterial activity, either (>400 μg/ml) (Table 1). The deacetylated form (**17**) of (**12**) did not exhibit antibacterial activity (Table 1). It suggests that hydrophobic nature of the compound could play an important role in an integration of the compound to the thick lipidic cell wall of bacilli.

In conclusion, the allyl group at C-1 of manninotrioside is critical for antibacterial activity. The acetylation of all the hydroxyl groups of β-manninotrioside is also critical for the antibacterial effect. One of the compounds with an allyl group, allyl isothiocyanate (AITC), is found in plants and AITC was reported to be mutagenic[3,4] and clastogenic,[5] while other reports indicated no genotoxic activity in this compound.[6–8] Wei and his co-workers also reported that not only allyl but also methyl isothiocyanate exhibit bactericidal activity against a rifampicin-resistant strain of *Salmonella* Montevideo, streptomycin-resistant strains of *Escherichia coli* O157:H7 and *Listeria monocytogenesis* Scott A.[9] Other groups of researchers reported that modification of penam sulfones with an allyl group rendered the compounds to be inhibitors of β-lactamase.[10] However, neither allyl alcohol (>400 μg/ml) nor AITC (>800 μg/ml) exhibited antibacterial activity against *M. tuberculosis* $H_{37}Rv$ and *S. aureus* (Table 1). These findings strongly suggest that allyl β-manninotrioside (OCT359) (**12**) exerts antibacterial activity by a mechanism distinct from that of AITC. The cell wall architecture of mycobacteria is only partly known. Daffe et al. reported that oligosaccharide fragments, **5**-Gal-(1 → 6)-Gal-(1 → 5)-Gal or **5**-Gal-(1 → 6)-Gal, were components in the cell wall of *M. tuberculosis*.[11] But manninotriose structure



Scheme 2. Chemical modification of C-1 site of β-manninotrioside.[12]

Table 1. Antibacterial effects of manninotriose derivatives (MIC, μg/ml)[a]

Compound	Organisms						
	M. tuberculosis $H_{37}Rv$	*M. avium* 724S	*S. aureus*	MRSA 873	MRSA 906	MRSA 908	MRSA 910
4	>800	ne	>800	ne	ne	ne	ne
5	>800	ne	>800	ne	ne	ne	ne
7	>800	ne	>800	ne	ne	ne	ne
8	>800	ne	>800	ne	ne	ne	ne
9	>800	ne	>800	ne	ne	ne	ne
10	>800	ne	>800	ne	ne	ne	ne
11	>800	ne	>800	ne	ne	ne	ne
12	3.13	3.13	3.13	3.77	15.1	7.54	15.1
13	>400	ne	>400	ne	ne	ne	ne
14	>800	ne	>800	ne	ne	ne	ne
16	>800	ne	>800	ne	ne	ne	ne
17	>100	>100	>100	100	>100	>100	>100
Allyl alcohol	>400	ne	>400	ne	ne	ne	ne
AITC	>800	ne	>800	ne	ne	ne	ne
Anti-MTB Antibiotics							
INH	0.075	12.5	>100	>100	>100	>100	>100
RIP	0.008	0.004	0.004	0.004	0.004	0.004	0.004
STM	0.20	0.40	100	>100	100	100	50
EMB	1.25	3.00	>100	>100	>100	>100	>100

[a] Broth dilution methods[13] using MiddleBrook 7H9 broth containing albumin, dextrose, and catalase for derivatives (ne, not examined). For *Staphylococcus aureus*, we used the heart-infusion broth. INH, isoniazide; RIF, rifampicin; STM, streptomycin; EMB, ethanbutol.

Table 2. Antimycobacterial effects of OCT359 on drug-sensitive and resistant clinical isolates of *M. tuberculosis*

Clinical isolates	Resistance to	MIC for OCT359 (μg/ml)
Drug-susceptive strains		
A-1-1		6.25
A-1-2		6.25
A-1-3		6.25
A-2-5		12.5
A-3-1		25
A-3-2		25
A-3-5		6.25
A-3-6		6.25
A-3-9		3.13
A-3-11		3.13
A-3-12		6.25
A-3-15		3.13
A-3-16		6.25
A-3-17		3.13
A-3-19		3.13
A-3-20		6.25
A-3-21		6.25
A-3-22		3.13
Drug-resistant strains		
A-2-1	INH	3.13
A-2-3	INH, RIF	3.13
A-2-4	RIF	12.5
A-2-6	INH, RIF, STR, EMB	6.25
A-3-47	STR	3.13
A-4-8	STR	6.25
A-4-25	INH, STR	12.5
A-4-30	INH, RIF, EMB	6.25
C-1-29	INH, RIF, STR, EMB	12.5
E-1-40	INH, RIF, STR, EMB	6.25
J-1-19	INH, RIF, EMB	3.13
K-3-6	INH	3.13
M-1-32	INH, RIF, STR, EMB	3.13
N-4-11	INH, RIF, EMB	3.13
N-5-2	INH, RIF, STR, EMB	3.13
P-1-50	INH, RIF, STR, EMB	3.13
P-4-11	INH, RIF, STR, EMB	3.13
Q-4-1	INH, RIF, STR, EMB	6.25
R-1-38	INH, RIF, STR, EMB	6.25
S-1-14	INH, RIF, STR, EMB	6.25
U-2-15	INH, RIF, STR, EMB	6.25
U-4-6	INH, RIF, STR, EMB	1.56
V-1-16	INH, RIF, STR, EMB	6.25
Z-1-2	INH, RIF, STR, EMB	3.13
Z-1-4	INH, RIF, STR, EMB	6.25
Reference strain		
M. tuberculosis $H_{37}Rv$		3.13

Proportion methods using Middlebrook 7H11 agar plates for INH (isoniazide), RIF (rifampicin), STR(streptomycin), EMB (ethambutol) and 7H9 broth for OCT359. Cut off concentrations of each antibiotic were 10, 10, 100, and 100 μg/ml, respectively.

is not known. But, OCT359 may show anti-bacterial activity by inhibiting the glycosyltransferases of bacilli. It should be noted that 25 clinical isolates of drug-resistant MTB and 19 drug-sensitive MTB were sensitive to OCT359. The MICs of OCT359 with these clinical isolates were from 3.13 to 25 μg/ml. The results strongly indicate that OCT359 could be a useful anti-bacterial compound against *M. tuberculosis*, *M. avium*, and *S. aureus*.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.02.024.

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12. General preparation procedures for alkyl *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1 → 6)-*O*-(2,3,4-tri-*O*-acetyl-α-D-galactopyranosyl)-(1 → 6)-*O*-2,3,4-tri-*O*-acetyl-β-D-glucopyranosides (alkyl 2,2′,2″,3,3′,3″,4,4′,4″,6″-deca-*O*-acetyl-β-manninotriosides). A suspension of *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galacto-pyranosyl)-(1 → 6)-*O*-(2,3,4-tri-*O*-acetyl-α-D-galacto-pyranosyl)-(1 → 6)-*O*-2,3,4-tri-*O*-acetyl-α-D-gluco-pyranosyl bromide (2,2′,2″,3,3′,3″,4,4′,4″,6″-deca-*O*-acetyl-α-mannino-triosyl bromide) (**6**) (5 g, 5.1 mmol), yellow mercuric (II) oxide (0.91 g, 4.2 mmol), mercuric (II) bromide (0.10 g, 0.3 mmol), drierite (1.3 g), and an appropriate anhydrous alcohol (35 ml) in purified $CHCl_3$ (35 ml) was stirred overnight at room temperature. The mixture was filtered through Celite and the filtrate was concentrated to dryness to afford a syrup or a solid which was dissolved in $CHCl_3$, filtered through Celite to remove insoluble mercuric salts, and concentrated to dryness to afford a thick syrup. The syrup was dissolved in a small amount of $CHCl_3$ and chromatographed on a column of silica gel, which was eluted with $CHCl_3$–acetone gradient (50:1–3:1, v/v). Evaporation of

the elute provided alkyl β-glycoside as an amorphous powder in 61–94% yields. The appropriate alcohols used were:methanol, ethanol, *n*-propyl alcohol, *i*-propyl alcohol, *n*-butyl alcohol, allyl alcohol, propargyl alcohol, and crotyl alcohol. Methyl β-glycoside (**7**): 74%, an amorphous powder, $[\alpha]_D^{22}$ + 118.3° (*c* 1.09,$CHCl_3$) lit. (2) an amorphous powder, $[\alpha]_D^{19}$ + 128.2° (*c* 1.70, $CHCl_3$), IR (Nujol) cm^{-1}:1750 (C=O). ^1H NMR ($CDCl_3$) δ: 1.98, 2.01, 2.05 (×2), 2.07 (×3), 2.08, 2.11, 2.14 (s, 30H, OAcx10), 3.53 (s, 3H, OMe), 4.45 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1), 4.97(d, 1H, $J_{1',2'or1'',2''}$ = 4.0 Hz, H-1′ or H-1″) and 5.10 (d, 1H, $J_{1',2'or1'',2''}$ = 4.3 Hz, H-1′ or H-1″). Ethyl β-glycoside (**8**): 95%, an amorphous powder, $[\alpha]_D^{22}$ + 120.5° (*c* 1.15, $CHCl_3$), IR (Nujol) cm^{-1}:1750 (C=O). ^1H NMR ($CDCl_3$) δ: 1.21(t, 3H, *J* = 7.0 Hz, OCH_2CH_3), 1.98, 2.01, 2.05 (×2), 2.07 (×2), 2.08, 2.11, 2.13, 2.14 (s, 30H, OAcx10), 4.53(d, 1H, $J_{1,2}$ = 8.2 Hz, H-1), 4.97(d, 1H, $J_{1',2'or1'',2''}$ = 3.4 Hz, H-1′ or H-1″) and 5.10 (d, 1H, $J_{1',2'or1'',2''}$ = 3.4 Hz, H-1′ or H-1″). Anal. Calcd for $C_{40}H_{56}O_{26}$: C, 50.42; H, 5.92. Found: C, 50.50; H, 6.00. *n*-Propyl β-glycoside (**9**):68%, an amorphous powder, $[\alpha]_D^{23}$ + 116.8° (*c* 1.01,$CHCl_3$), IR (Nujol) cm^{-1}:1750 (C=O). ^1H NMR ($CDCl_3$) δ: 0.91 (t, 3H, *J* = 7.5Hz, $OCH_2CH_2CH_3$), 1.60 (m, 2H, $OCH_2CH_2CH_3$), 1.98 (×2), 2.01, 2.04, 2.06, 2.08 (×2), 2.12, 2.14, 2.15 (s, 30H, OAcx10), 3.47, 3.80(m, 2H, $OCH_2CH_2CH_3$), 4.05 (dd, 1H, $J_{5,6a}$ = 7.3 Hz, $J_{6a,6b}$ = 11.3 Hz, H-6a), 4.17 (dd, 1H, $J_{5,6b}$ = 5.8 Hz, H-6b), 4.50 (d, 1H, $J_{1,2}$ = 8.2 Hz, H-1), 4.95 (d and dd, 2H, $J_{2,3}$ = 9.1 Hz, $J_{1',2'or1'',2''}$ = 4.3 Hz, H-2, H-1′ or H-1″), and 5.16 (d, 1H, $J_{1',2'or1'',2''}$ = 3.7 Hz, H-1′ or H-1″). Anal. Calcd for $C_{41}H_{58}O_{26}$: C, 50.93; H, 6.05. Found:C, 50.88; H, 6.09. *i*-Propyl β-glycoside (**10**):77%, an amorphous powder, $[\alpha]_D^{22}$ + 111.8° (*c* 1.17, $CHCl_3$), IR (Nujol) cm^{-1}: 1750 (C=O). ^1H NMR ($CDCl_3$) δ: 1.15, 1.24 (each d, 6H, $CH(CH_3)_2$), 1.98 (×2), 2.01, 2.04, 2.06, 2.09 (×2), 2.12, 2.14, 2.15 (s, 30H, OAcx10), 3.93 (m, 1H, $CH(CH_3)_2$), 4.05 (dd, 1H, $J_{5,6a}$ = 7.3 Hz, $J_{6a,6b}$ = 11.3 Hz, H-6a), 4.17 (dd, 1H, $J_{5,6b}$ = 5.8 Hz, H-6b), 4.56 (d, 1H, $J_{1,2}$ = 8.2 Hz, H-1), 4.90 (dd, 1H, $J_{2,3}$ = 9.4 Hz, H-2), 4.95(d, 1H, $J_{1',2'or1'',2''}$ = 3.4 Hz, H-1′ or H-1″), and 5.15 (d, 2H,$J_{1',2'or1'',2''}$ = 3.4 Hz, H-1′ or H-1″). Anal. Calcd for $C_{41}H_{58}O_{26}$: C, 50.93; H, 6.05. Found: C, 50.76; H, 6.02. *n*-Butyl β-glycoside (**11**): 78%, an amorphous powder, $[\alpha]_D^{22}$ + 127.0° (*c* 0.74, $CHCl_3$), IR (Nujol) cm^{-1}: 1750 (C=O). ^1H NMR ($CDCl_3$) δ: 0.91 (t, 3H, *J* = 7.3Hz, $OCH_2CH_2CH_2CH_3$), 1.35 (m, 2H, $OCH_2CH_2CH_2CH_3$), 1.56 (m, 2H, $OCH_2CH_2CH_2CH_3$), 1.98 (×2), 2.01, 2.04, 2.06, 2.08 (×2), 2.12, 2.14 (×2) (s, 30H, OAcx10), 3.50, 3.85 (m, 2H, $OCH_2CH_2CH_2CH_3$), 4.04 (dd, 1H, $J_{5,6a}$ = 7.3 Hz, $J_{6a,6b}$ = 11.3 Hz, H-6a), 4.17 (dd, 1H, $J_{5,6b}$ = 5.5 Hz, H-6b), 4.50 (d, 1H, $J_{1,2}$ = 8.2 Hz, H-1), 4.94 (dd, 1H, $J_{2,3}$ = 8.9Hz, H-2), 4.95(d, 1H, $J_{1',2'or1'',2''}$ = 3.7 Hz, H-1′ or H-1″) and 5.16 (d, 1H, $J_{1',2'or1'',2''}$ = 3.4 Hz, H-1′ or H-1″). Anal. Calcd for $C_{42}H_{60}O_{26}$: C, 51.43; H, 6.17. Found: C, 51.22; H, 6.18. Allyl β-glycoside (**12**), OCT359: 84%, an amorphous powder, $[\alpha]_D^{23}$ + 123.6° (*c* 1.01, $CHCl_3$), IR (Nujol) cm^{-1}: 1750 (C=O), 1648 (C=C). ^1H NMR ($CDCl_3$) δ: 1.98 (×2), 2.01, 2.05, 2.06, 2.08, 2.09, 2.12, 2.14 (×2) (s, 30H, OAcx10), 4.05 (dd, 1H, $J_{5,6a}$ = 7.4 Hz, $J_{6a,6b}$ = 11.3 Hz, H-6a), 4.13, 4.33(m, 2H, $OCH_2CH{=}CH_2$), 4.18 (dd, 1H, $J_{5,6b}$ = 5.5 Hz, H-6b), 4.57 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1), 4.96(d, 1H, $J_{1',2'or1'',2''}$ = 3.7 Hz, H-1′ or H-1″), 5.17 (d, 1H, $J_{1',2'or1'',2''}$ = 4.3 Hz, H-1′ or H-1″), and 5.87 (m,1H, $OCH_2CH{=}CH_2$). Anal. Calcd for $C_{41}H_{56}O_{26}{\cdot}H_2O$: C, 50.10; H, 5.95. Found: C, 50.19; H, 5.95. Propargyl β-glycoside (**13**): 81%, an amorphous powder, $[\alpha]_D^{22}$ + 117.8° (*c* 1.07, $CHCl_3$), IR (Nujol) cm^{-1}: 1750 (C=O), 2150 (C≡C). ^1H NMR ($CDCl_3$) δ: 1.98 (×2), 2.01, 2.05, 2.06, 2.07, 2.09, 2.11, 2.13 (×2) (s,30H, OAcx10), 2.59 (t, 1H, *J* = 2.4 Hz, $OCH_2C{\equiv}CH$), 4.39, 4.44 (dd, 2H, *J* = 15.9Hz, $OCH_2C{\equiv}CH$), 4.80 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1), 4.96 (d, 1H, $J_{1',2'or1'',2''}$ = 3.1 Hz, H-1′ or H-1″), and 5.16 (d, 1H, $J_{1',2'or1'',2''}$ = 3.4 Hz, H-1′ or H-1″). Anal. Calcd for $C_{41}H_{54}O_{26}$: C, 51.14; H, 5.65. Found: C, 50.85; H, 5.60. Crotyl β-glycoside (**14**): 61%, an amorphous powder, $[\alpha]_D^{22}$ + 120.0° (*c* 1.10, $CHCl_3$), IR (Nujol) cm^{-1}: 1750 (C=O), 1648 (C=C). ^1H NMR ($CDCl_3$) δ: 1.73 (dd, 3H, *J* = 6.7 Hz, *J* = 0.9 Hz, $OCH_2CH{=}CHCH_3$), 1.98 (×2), 2.01, 2.04, 2.05, 2.08 (×2), 2.12, 2.13, 2.14 (s, 30H, OAcx10), 4.04, 4.25 (m, 2H,$OCH_2CH{=}CHCH_3$), 4.56 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1), 4.96 (d, 1H, $J_{1',2'or1'',2''}$ = 3.7 Hz, H-1′ or H-1″), 5.17 (d, 1H, $J_{1',2'or1'',2''}$ = 3.7 Hz, H-1′ or H-1″), 5.50 (m, 1H, $OCH_2CH{=}CHCH_3$), and 5.74 (m, 1H, $OCH_2CH{=}CHCH_3$). Anal. Calcd for $C_{42}H_{58}O_{26}$: C, 51.53; H, 5.97. Found: C, 51.27; H, 5.98.

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- 納品書
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瀧　井　猛　将

Dihydrotestosterone Inhibits Tumor Necrosis Factor α Induced *Interleukin-1α* mRNA Expression in Rheumatoid Fibroblast-Like Synovial Cells

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Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects multiple synovial joints. Proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF)α play important roles as principle inflammatory and destructive components of the disease. RA is known to be associated with significant gender differences in its prevalence and clinical features. We found that a potent androgen, 5α-dihydrotestosterone (DHT) inhibits *IL-1α* mRNA expression induced by TNFα and the DHT effect was inhibited by an androgen receptor antagonist, hydroxyflutamide (OHF). DHT inhibited the NF-κB activation induced by TNFα in a manner dependent on the androgen receptor (AR). These results suggest that DHT inhibits the TNFα-induced *IL-1α* mRNA expression by inhibiting NF-κB activation, and contributes to the gender differences of the disease.

Key words rheumatoid arthritis; synoviocytes; interleukin-1 (IL-1); tumor necrosis factor (TNF)α; androgen; androgen receptor

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by the chronic inflammation and hyperproliferation of synovial cells in multiple joints. Proinflammatory cytokines and chemokines produced by synoviocytes and infiltrated immune cells are implicated in the disease pathogenesis, such as production of proteases and reactive oxygen intermediates, proliferation of synovial fibroblasts, cartilage degradation, infiltration of inflammatory cells and angiogenesis.[1] Antibody against TNFα, soluble receptor for TNFα, interleukin-1 receptor antagonist (IL-1ra) and antibody against IL-6 appeared to be effective in the treatment of RA patients, indicating that these cytokines are pivotal in the pathogenesis of the disease.[2—5] These cytokines form a network, such as the induction of IL-1 and IL-6 by TNFα, and IL-6 induction by IL-1.[6] Fibroblast-like synoviocytes are major cells that produce IL-1 and IL-6, and TNFα is probably produced by macrophages.[7]

There are two types of IL-1, IL-1α and IL-1β. They bind to the same receptor, type I IL-1 receptor, and transduce intracellular signaling.[6] IL-1 contributes to the pathogenesis of the disease by affecting fibroblast-like synovial cells and cartilage to induce matrix metalloproteases (MMPs), NO and prostaglandin E2, which accelerate cartilage degradation. IL-1 also inhibits the synthesis of type II collagen from cartilage.[8—10] By the experiments using animal model of arthritis IL-1α appeared to be important in the early phase of arthritis and participates in the inhibition of proteoglycan synthesis.[11,12]

Although it is not clarified how and what causes RA, RA is deviated to women as also observed in other autoimmune diseases; the ratio of RA incidence in women to men is about 4 : 1. The levels of estrogen to androgen in synovial fluid are elevated in both male and female RA patients[13] and the level of testosterone in serum and synovial fluid is lower in male RA patients as compared to normal individuals.[14] Therefore, sex hormones are implicated in the gender difference of RA.

In this study, we examined the effect of 5α-dihydrotestosterone (DHT) on the induction of *IL-1α* mRNA by TNFα, and suggested that DHA inhibits the effect of TNFα by inhibiting NF-κB activation in a manner dependent on the androgen receptor (AR).

MATERIALS AND METHODS

Reagents Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); fetal bovine serum (FBS) was from JRH Bioscience (Lenexa, KS, U.S.A.); 5α-dihidrotestosterone (DHT) was from Fluka (Buchs, Switzerland). Hydroxyflutamide (OHF) was kindly provided by Nippon Kayaku Co., Ltd. (Tokyo, Japan). Human recombinant TNFα was provided by Dainippon Pharmaceutical Co. (Osaka, Japan). The specific activity of TNFα was 2.55×10^6 U/mg based on the cytotoxic assay using L929 cells cultured with actinomycin D.

Cell Culture Primary synovial cells from RA patients as well as MH7A, an immortalized cell line established by stably transfecting rheumatoid synoviocytes with the SV40 T antigen gene,[15] were cultured in DMEM, with 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 4 mM L-glutamine and 10% heat-inactivated FBS at 37 °C in air containing 5% CO_2. This study has been approved by the ethics committee of Kitasato University and Nagoya City University.

Plasmids pGL3-κBwt and NF-κB mutant construct

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pGL3-κBm (for NF-κB reporter gene assay) have been described previously.[16] pcDNA3-F-AR (AR expression vector) was constructed as described previously.[17]

Transient Transfection and Luciferase Assays The day before transient transfection of MH7A cells, the culture medium was replaced by phenol red-free DMEM supplemented with 10% heat-inactivated FBS that had been pretreated with dextran-coated charcoal to remove endogenous sex hormones. NF-κB reporter plasmid, AR expression plasmid and pCMV-βgal plasmid (for normalization of transfection efficiency) were transiently transfected into MH7A cells using the calcium phosphate-DNA co-precipitation method. After 16 h of transfection, cells were incubated with DHT for an additional 24 h and harvested. Luciferase assays were performed with the luciferase reporter gene assay kit (Roche, Germany) according to the manufacturer's instructions. The light emission was measured using a multilabel counter 1420 ARVO (Perkin Elmer, Wellesley, MA, U.S.A.). Luciferase activity was expressed after normalization with the β-galactosidase value in the same sample.

Semiquantitive Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis Total RNA from cells was extracted according to the method of Chomczynski and Sacchi.[18] The RT-PCR analysis was performed as described previously.[19] Two micrograms of total RNA were reverse-transcribed with 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP, 40 ng random primer, p(dN)6, 6 U ribonuclease inhibitor, and 40 U Maloney murine leukemia virus (MMLV) RT. One-tenth of the reversely-transcribed materials was used in the PCR reactions. Primers used for human *IL-1α* were 5′-ATGGCCAAAGTTCCAGACATG-3′ and 5′-CTACGCCTGGTTTTCCAGTATCTGAAAGTCAGT-3′ (816-bp product); for human *GAPDH* were 5′-TGAAGGTCGGAGTCAACGGATTTGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (980-bp product). PCR were conducted as following conditions; 94 °C, 10 min; 30 cycles (human *IL-1α* or 24 cycles (human *GAPDH*) of 94 °C for 1 min, 54 or 56 °C for 1 min and 72 °C 1 min.

RESULTS

DHT Inhibits the TNFα-Induced *IL-1α* mRNA Expression in Rheumatoid Synoviocytes To investigate the effects of TNFα and DHT on the *IL-1α* mRNA expression in RA synovial cells, three primary preparations of fibroblast-like synovial cells from RA patients were treated with or without TNFα in the presence or absence of physiological concentration of DHT; serum level of total testosterone is 20.10±4.50 and 19.10±4.50 nmol/l in normal men and RA men, respectively. In women, serum testosterone is 0.78±0.27 normal women and 0.20±0.06 nmol/l in RA patients, respectively.[20] RT-PCR analysis indicated that TNFα induced *IL-1α* mRNA expression in these primary synovial cells, and DHT significantly inhibited the *IL-1α* mRNA inducing activity of TNFα (Fig. 1).

Androgen Receptor Antagonist Recovers the DHT Inhibition of TNFα-Induced *IL-1α* mRNA Expression We also determined the effects of TNFα and DHT on the expression of *IL-1α* mRNA in fibroblast-like synovial cell line MH7A. Similarly to primary synovial cells TNFα at either 10 or 1 U/ml induced *IL-1α* mRNA expression in MH7A cells and DHT inhibited the effect of TNFα (Fig. 2). To determine whether the inhibitory effect of DHT was mediated by AR, MH7A cells were treated with TNFα with or without DHT in the presence or absence of androgen receptor antago-

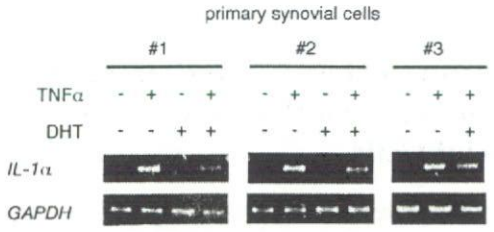

Fig. 1. Effect of DHT on the TNFα-induced *IL-1α* mRNA Expression in Rheumatoid Synoviocytes

Primary synovial cells were treated with or without 10 U/ml of TNFα in the presence or absence of 10 nM DHT for 8 h. Total RNA was extracted, and expression levels of mRNA of *IL-1α* and *GAPDH* were determined by RT-PCR method. Representative data of two independent experiments with similar results were shown.

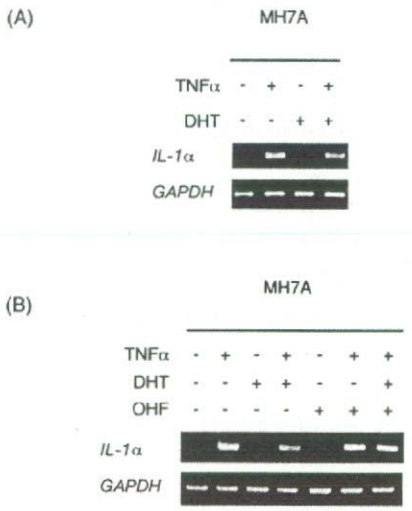

Fig. 2. Effect of Androgen Receptor Antagonist on the DHT Inhibition of TNFα-Induced *IL-1α* mRNA Expression in MH7A Cells

MH7A cells were treated with or without TNFα at 10 U/ml (A) or 1 U/ml (B) in the presence or absence of 10 nM DHT and 2 μM OHF for 8 h. Total RNA was extracted, and expression levels of mRNA of *IL-1α* and *GAPDH* were determined by RT-PCR method. Representative data of three independent experiments with similar results were shown.

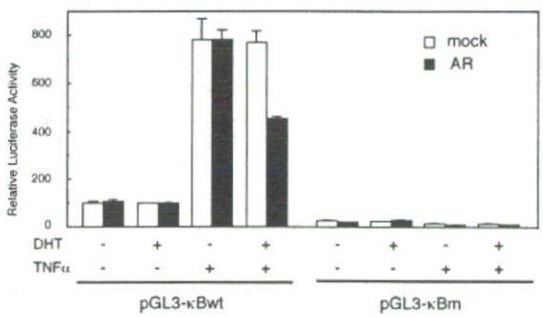

Fig. 3. Effect of DHT on the TNFα-Induced NF-κB Activation

MH7A cells were transiently transfected with pGL3-κBwt, pGL3-κBm, pcDNA3-F-AR and pCMV-βgal. After 16 h, cells were treated with or without 10 nM DHT in the presence or absence of 10 U/ml of TNFα for 24 h and luciferase activity was measured. The luciferase activity was normalized by β-galactosidase activity.

nist, hydroxyflutamide (OHF).[21] As shown in Fig. 2B, OHF significantly reversed the inhibitory effect of DHT.

DHT Inhibits the TNFα-Induced NF-κB Activation in a Manner Dependent on AR NF-κB is a key transcription factor for induction of IL-1α mRNA.[22] To determine whether DHT inhibits the NF-κB activation by TNFα *via* the AR, MH7A cells were transfected with the NF-κB-dependent reporter plasmid with or without the expression vector of AR, and treated with TNFα in the presence or absence of DHT. As shown in Fig. 3, TNFα induced luciferase activity from the reporter plasmid containing four wild type NF-κB binding sites, but not the reporter plasmid containing four mutated inactive NF-κB binding sites. DHT alone did not affect the NF-κB activity and DHT did not inhibit the TNFα-induced NF-κB activation without AR overexpression. However, in the presence of over-expressed AR, DHT significantly inhibited the TNFα-induced NF-κB activation.

DISCUSSION

This is the first report indicating the inhibitory effect of DHT on *IL-1α* mRNA expression induced by TNFα in fibroblast-like synoviocytes derived from RA patients and fibroblast-like synoviocyte line MH7A. The inhibitory effect of DHT was recovered by androgen receptor antagonist. In addition, the reporter gene assay revealed that NF-κB activation by TNFα was inhibited by DHT in the presence of over-expressed AR. We confirmed AR expression in synovial cells and MH7A (data not shown). As the effect of DHT was not observed in the absence of over-expressed AR, the expression level of endogenous AR may not be sufficient to exhibit its function against transfected reporter plasmid. NF-κB is a critical transcription factor for activation of the *IL-1α* gene.[22] These results suggest that the inhibitory effect of DHT is due to the inhibition of NF-κB activation by TNFα *via* AR, although we do not rule out the possibility that other transcription factors are also affected by DHT/AR.

NF-κB forms a dimer with Rel family proteins and is maintained in the cytoplasm as inactive complexes with inhibitory proteins, called IκBs. Stimulation of TNFα or IL-1 leads to the sequential activation of the adapter protein myeloid differentiation factor 88 (MyD88), the IL-1 receptor associated kinases (IRAKs), TRAF2 or TRAF6, and eventually, the IκB kinase complex (IKKα, β, γ). The IKK complex phosphorylates the IκBs, targeting them for ubiquitination and degradation by the proteasome. Degradation of IκB liberates NF-κB/Rel dimmers which translocate to the nucleus and augment the expression of NF-κB-responsive genes, including, IL-1α, IL-1β, IL-8, TNFα, MMPs and adhesion molecules.[23]

AR is a member of the group of four closely related steroid receptors, the other members of which are glucocorticoid receptor, mineralocorticoid receptor, and progesterone receptor.[24] This group comprises a subfamily of the larger and more diverse family of nuclear transcription factors. AR can be divided into four domains, an N-terminal domain that is involved in transcriptional regulation, a DNA-binding domain, a hinge region and a C-terminal ligand-binding domain. The ligand penetrates into cells and binds to AR in the cytoplasm. The ligand-activated AR forms a homodimer, translocates into nucleus, binds to target DNA, interacts with coactivators and induces transcription of the genes.

The mechanism by which the DHT/AR inhibits NF-κB activation is unclear. In COS-1 cells elevated expression of RelA (p65) repressed AR-mediated transactivation accompanied by the weak interaction between AR and RelA,[25] and in prostate cancer LNCaP cells DHT suppressed NF-κB activity accompanied by a slight increase of IκB level.[26] In a preliminary study we observed that GHA/AR inhibited the TNFα-induced degradation of IκB. However, the precise mechanism how DHT/AR inhibit the NF-κB activation by TNFα in synovial cells remains to be elucidated.

We have found that estrogen augments the mRNA expression of IL-1α by activating transcription of the gene *via* estrogen receptor α.[27] By treatments with IL-1ra or antibodies against IL-1α or IL-1β, both IL-1α and IL-1β appeared to contribute to the arthritis caused by the immunization with type II collagen or by immune complex in the mouse.[28] In IL-1α transgenic mouse membrane-bound IL-1α from synoviocytes appeared to be critical for the induction of arthritis.[12] Interestingly, the severity of arthritis was correlated with membrane bound IL-1α rather than serum IL-1α or IL-1β. Therefore, our study suggests that DHT is a negative regulator for the induction or pathogenesis of RA by inhibiting TNFα-mediated induction of IL-1α and contributes to the gender differences of RA.

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The interaction with Sp1 and reduction in the activity of histone deacetylase 1 are critical for the constitutive gene expression of IL-1α in human melanoma cells

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Abstract: A375-6 human melanoma cells are sensitive to the antiproliferative effect of IL-1. After a long period of culturing, we have obtained cells resistant to IL-1. The resistant clone A375-R8 constitutively produced IL-1α. In this study, we identified a sequence, CGCC, located at −48 to −45 upstream of the transcription start site, to be essential for the constitutive IL-1α gene activation. Specificity protein 1 (Sp1) and Sp3 bound to the nucleotide containing the sequence. Although the binding level to the nucleotide and expression level of Sp1 and Sp3 are comparable in A375-R8 and A375-6 cells, transactivation activity of Sp1 is higher in A375-R8 cells as compared with A375-6 cells. Sp3 could not transactivate the IL-1α promoter. These results suggest that Sp1 but not Sp3 is important for IL-1α gene activation. Trichostatin A (TSA), an inhibitor of histone deacetylase (HDAC), greatly augmented the IL-1α promoter activity in A375-6 cells to the level comparable with that in A375-R8 cells. TSA also induced IL-1α mRNA expression in A375-6 cells. Sp1 and Sp3 bound to HDAC1 in A375-R8 and A375-6 cells. The chromatin immunoprecipitation assay revealed the binding of Sp1 and HDAC1 to the promoter region of the IL-1α gene. The activities of HDAC bound to Sp1 and Sp3, and that of HDAC1 was lower in A375-R8 cells as compared with A375-6 cells. These results indicate that the reduction in the activity and interaction of HDAC1 with Sp1 are critical for the constitutive IL-1α gene expression. *J. Leukoc. Biol.* 83: 190–199; 2008.

Key Words: *HDAC · trichostatin A · chromatin immunoprecipitation*

INTRODUCTION

IL-1 plays an important role in a variety of pathophysiological reactions, including immunologic and inflammatory reactions. IL-1 also functions as a growth regulatory molecule by acting positively or negatively [1]. There are two types of IL-1, α and β, and their genes share a similar intron/exon structure. Although they share the same biological activities by binding to the same receptor, type I receptor (IL-1RI), the precursor of IL-1β binds weakly to IL-1RI and cannot exert biological activity, and that of IL-1α can bind to IL-1RI and exerts biological activity. Therefore, IL-1β works only after proteolytic maturation followed by the release from its producing cells, and IL-1α can exert biological activity in precursor and mature forms. IL-1α is also active in a membrane-bound form.

The gene activation mechanism for human IL-1β has been studied extensively. Studies revealed that the C/EBPβ/NF-IL-6 site, NF-β1 site, cAMP response element-like site, AP-1 site, and NF-κB site are important for activation of the IL-1β gene [2, 3]. In contrast, the regulatory mechanism of the IL-1α gene expression is largely unknown [4]. In macrophages and monocytes, IL-1α and -β are often induced by the same stimuli [1]. However, in other cell types, including keratinocytes [5], endothelial cells [6], T cells [7], melanoma cells [8], and ovarian tumor cells [9], gene expression of these two IL-1s appears to be regulated rather independently. In addition, sustained production of IL-1α is observed and is implicated in many chronic diseases, including juvenile rheumatoid arthritis [10], inflammatory myopathies [11], scleroderma [12], cystic fibrosis [13], HIV infection [14], gastric carcinoma [15], and ovarian cancer [16]. Constitutively produced IL-1α is an autocrine growth factor for Kaposi sarcoma [17] and induces senescence of endothelial cells [6] and fibroblasts [18].

In various experimental models, IL-1 increases tumor invasiveness and metastasis [1, 19] Melanoma cells in culture produce a variety of growth factors and cytokines, including IL-1, IL-6, and TGF-α and -β [20]. IL-1 is implicated in the pathogenesis of melanoma through effects on its own producing cells, adjacent cells, and infiltrating lymphoid cells. IL-1 also exhibits systemic effects, including fever, diarrhea, anorexia, somnolence, and hypercalcemia [1]. The acquired resistance against antiproliferative cytokines IL-1, IL-6, and oncostatin M

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is implicated in malignancy of human melanoma [20, 21]. In addition, some melanoma cell lines established from a human melanoma legion produce IL-1 constitutively [11].

We have reported that IL-1 is antiproliferative for a human melanoma cell line (A375-6) [22, 23]. During the study, we noticed that the IL-1-sensitive cells became resistant to IL-1, although they expressed functional IL-1RI [24]. The IL-1-resistant clone, A375-R8, constitutively produced IL-1α but not IL-1β in protein and mRNA levels. As the resistant cells exhibited many features of advanced melanoma, including augmented expression of adhesion molecules and production of cytokines and matrix metalloproteinase [25], it was suggested that the IL-1 resistance is associated with the malignant phenotype of melanoma. We reported previously that IL-1α stimulates its own gene expression and production through activation of NF-κB in an autocrine manner [26]. We also noticed that there is a region in the IL-1α promoter responsible for the constitutive gene activation. In this study, we analyzed the molecular mechanism of the constitutive activation of the IL-1α gene using IL-1α-nonproducing A375-6 cells and IL-1α-producing A375-R8 cells.

MATERIALS AND METHODS

Reagents

RPMI-1640 medium, Trichostatin A (TSA), and anti-β-actin mAb were purchased from Sigma Chemical Co. (St. Louis, MO, USA). FBS was obtained from HyClone (Logan, UT, USA); anti-specificity protein 1 (Sp1) rabbit antibody (sc-59) and anti-Sp3 rabbit antibody (sc-644) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-histone deacetylase 1 (HDAC1) mouse mAb (clone 2E10) from Upstate Biotechnology (Lake Placid, NY, USA); and antiphosphoserine rabbit antibody from Zymed Laboratories, Inc. (South San Francisco, CA, USA).

Plasmids

IL-1α genomic DNA fragments were cloned into pGL3-basic (Promega, Madison, WI, USA) to generate pGL3-IL1a(−421), pGL3-IL1a(−103), pGL3-IL1a(−70), pGL3-IL1a(−61), pGL3-IL1a(−53), pGL3-IL1a(−51), pGL3-IL1a(−31), and pGL3-(IL1a-GC)$_3$. pGL3-IL1a(−421)mATAA, replacing CGCC to ATAA at −48 to −45, and pGL3-IL1a(−421)ΔCC, deleting CC at positions −46 to −45, were generated by PCR. All constructs were verified by sequencing. pCIneo-Sp1, pAC-ΔNSp1, and pAC-ΔCSp1 were generous gifts from Dr. Soichi Kojima (Riken, Japan) [27] and pCMV4-Sp3 from Dr. Jonathan M. Horowitz (North Carolina State University, Raleigh, NC, USA) [28]. Galactosidase 4 (GAL4)-Sp3 was generated by PCR. pFR-luciferase (Luc; 5×GAL4-Luc) was purchased from Stratagene (La Jolla, CA, USA).

Cell cultures

The human melanoma cell line A375 was given originally by Dr. Raymond Ruddon (National Cancer Institute, Bethesda, MD, USA). By limiting dilution, a subclone, IL-1-sensitive A375-6, was obtained [23]. An IL-1-resistant subclone, A375-R8, was obtained by limiting dilution of A375-6 cells, which had acquired resistance to IL-1 after routine passage for 3 months [24]. These cells were cultured in RPMI-1640 medium supplemented with 15 mM HEPES and 5% heat-inactivated FBS.

Immunoprecipitations and Western blot analysis

After being transfected with the indicated plasmid, 1×10^6 cells were lysed in radioimmunoprecipitation assay (RIPA)-A buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, and 1% Triton-X 100), supplemented with protease inhibitors and phosphatase inhibitors. The lysates were then subjected to immunoprecipitation, and coimmunoprecipitates or 1–2% of lysates were subjected to SDS-PAGE (12.5%), transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and probed with antibodies described in the figure legends. For immunoprecipitation, 4 μg/20 μl lysates of anti-Sp1, anti-Sp3, or anti-HDAC 1 antibodies were used. For immunoblotting, 0.5 μg/ml anti-Sp1, anti-Sp3, anti-HDAC1, or anti-β-actin antibodies were used. The immunoreactive proteins were visualized using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA), and the light emission was quantified by a LAS-1000 lumino image analyzer (Fuji Film, Tokyo, Japan).

Transfection and luciferase assay

For IL-1α promoter analysis and pGL3-(IL1a-GC)$_3$ transcriptional assays, A375 cells were cotransfected with IL-1α promoter reporter plasmid or pGL3-(IL1a-GC)$_3$ reporter plasmid together with pCMV-β-gal and pCMV5 in the presence or absence of Sp1 or Sp3 expression plasmids (pCIneo-Sp1, pAC-ΔNSp1, pAC-ΔCSp1, and pCMV4-Sp3) [27, 28] by using Effectene transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For GAL-4-Sp1and -Sp3 transcriptional assays, the cells were cotransfected with the reporter plasmid pFR-Luc and GAL-4-Sp1 or -Sp3 construct with pCMV-β-gal and pCMV5. To 5×10^5 cells, 0.03 μg reporter plasmid, 0.02 μg pCMV-β-gal, and 0.15 μg other plasmids were transfected. In some experiments, 24 h after the transfection, the medium was replaced with medium, with or without TSA. After 24 h incubation, cell lysates were collected, and luciferase assay was performed with the luciferase reporter gene assay kit (Roche, Germany), according to the manufacturer's instructions. Luciferase activity was determined by using the β-gal value as a basis for normalization.

EMSA

Preparation of nuclear extracts and EMSA were carried out as described previously [29]. Samples of the nuclear extracts (15 μg) were analyzed by 6% nondenatured PAGE. For supershift assay, the nuclear extracts were incubated with 2 μg anti-Sp1 or anti-Sp3 antibodies before addition of the labeled probe. The sequences of the top strands of the individual oligonucleotide probe were as follows; 1, 5′-CGTAGCCACGCCTACTTAAG-3′; 2, 5′-TAGCCACGCCTACTTAAGAC-3′; 3, 5′-CCACGCCTACTTAAGACAAT-3′; 4, 5′-ACGCACTTGTAGCCACG-TAG-3′; m1, 5′-CGTAGCCAATAATACTTAAG-3′; m2, 5′-CGTATAAACGC-CTACTTAAG-3′. Competition experiments were performed with the corresponding unlabeled, double-stranded oligonucleotides.

RNA extraction and RT-PCR

Total RNA was extracted from A375 cells, and RT-PCR analysis was performed as described previously [24]. For RT-PCR analysis, total RNA from A375 cells was reverse-transcribed into cDNA, and the amplification reaction was carried out using sense and antisense primers for IL-1α and GAPDH as previously reported [30]. RT-PCR products were analyzed on a 1.5% agarose electrophoresis gel in the presence of ethidium bromide.

Chromatin immunoprecipitation (ChIP) assay

Formaldehyde was added directly to the cell culture medium to a final concentration of 1%. Fixation was carried out at room temperature for 10 min prior to quenching with 0.125 M glycine for 5 min. Cells containing 1×10^6 were lysed in Triton harvest buffer containing protease inhibitors, and nuclei were pelleted by centrifugation. The nuclear pellet was resuspended in RIPA-B buffer (10 mM Tris at pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and protease inhibitors) and sonicated to an average fragment length 500–1000 bp. The chromatin suspension was precleared by centrifugation and then incubated overnight with 4 μg anti-Sp1, anti-Sp3, or anti-HDAC 1 antibodies or a no-antibody control. Chromatin-antibody complex was purified on protein A- or G-Sepharose beads, which have been preblocked with 100 μg/ml sheared salmon sperm DNA. The beads were washed six times with RIPA-B buffer. After the elution of immunoprecipitates and the reversal of the cross-links by heating to 65°C for 4 h, the DNA was recovered by phenol/chloroform extraction and precipitated by ethanol. Then, the association of Sp1 and HDAC1 with the IL-1α promoter was measured by PCR. The primers used for the amplification of the core IL-1α promoter region were forward: TCGGATTTCACGATTTGTCC and reverse: ACGGGGAATTTACAGGGAAG.

HDAC assay

HDAC assay was performed using the HDAC assay kit (Upstate Biotechnology), according to the manufacturer's instructions. Briefly, 30 μl immunoprecipitated proteins were incubated with 20 μl [^{3}H]acetate-labeled Histone H4 peptide in a total volume of 200 μl for 24 h at room temperature. The reaction was stopped by addition of 50 μl quenching solution. After centrifugation at 14,000 *g* for 2 min, a 100-μl aliquot of the organic phase was counted in 5 ml liquid scintillation cocktail.

RESULTS

Regulatory regions responsible for the constitutive activation of the IL-1α gene

As shown in **Figure 1**, −421 upstream of the transcriptional start of the human IL-1α gene contained potential transcriptional control elements, including glucocorticoid responsive element (GRE)-like, NF-κB, GC box, TATA-like sequence, and AP-1 binding sites. In previous studies, we found that A375-R8 cells, but not A375-6 cells, constitutively express IL-1α mRNA and secrete an active IL-1α molecule [24]. IL-1β mRNA is not expressed in these cells. To analyze the promoter region involved in the constitutive activation of the IL-1α gene in A375-R8 cells, we constructed a series of 5′-end deletion mutants of the IL-1α gene with the luciferase expression plasmid. These reporter plasmids were transfected into A375-R8 and A375-6 cells, and the luciferase activity in the cell lysates was determined (Fig. 1A). As has been reported [26], in A375-R8 cells, deletion of the upstream sequence to the nucleotide position from −421 to −103 resulted in an augmentation of the activity. By further deletion to −70, the transcriptional activity was decreased substantially as compared with −103. The activity was decreased markedly by further deletion to −31. These results indicate that the sequence between −103 and −70 contained a positively regulatory element(s) in A375-R8 cells but not in A375-6 cells. The positively regulatory element(s) was also found in the sequence between −70 and −31. Similar, but less effective, responses were observed in A375-6 cells.

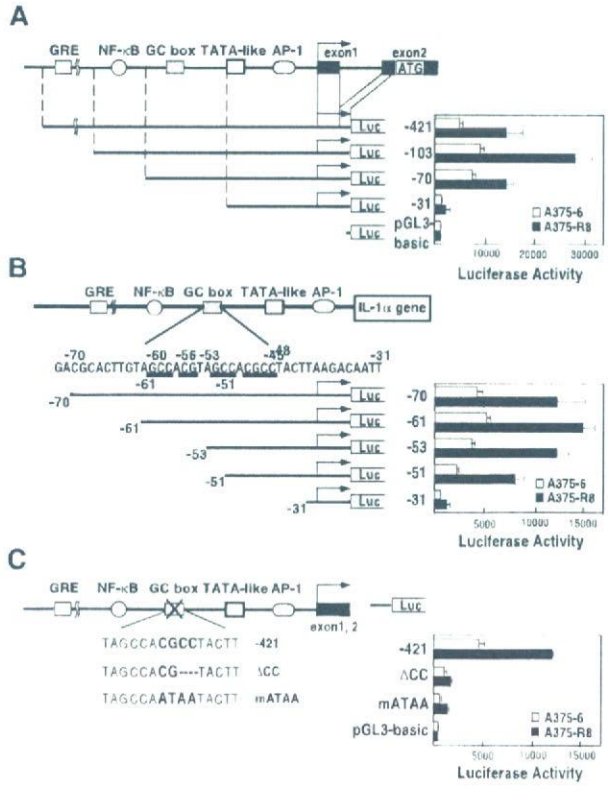

Fig. 1. Deletion analysis of the 5′-flanking region of the human IL-1α gene in A375 cells. (A–C) Putative consensus sequences in the 5′-upstream region of the human IL-1α gene are illustrated. Numbers indicate the distance in base pairs from the transcription start. Each reporter plasmid was transfected into A375 cell, which were harvested 48 h after transfection, and the luciferase activity was measured. After normalization with β-gal activity, the luciferase activity was indicated. Mean + SD based on triplicate cultures is shown.

We analyzed the promoter region between −70 and −31 further by constructing additional mutants (Fig. 1B). Compared with pGL3-IL-1a(−31), pGL3-IL-1a(−51) exhibited a higher luciferase activity in A375-R8 cells, suggesting a positively regulatory element(s) between −51 and −31. In addition, the sequence between −53 and −51, although less effectively, contributed to the IL-1α promoter activity. Similar, but less effective, responses were observed in A375-6 cells.

The region −51 and −31 contains a sequence CCCC (−48 to −45). To investigate the functional relevance of the sequence in the IL-1α promoter activity, we constructed mutant plasmids in the region by deletion [pGL3-IL-1a(421)ΔCC] or substitution [pGL3-IL-1a(421)mATAA]. As shown in Figure 1C, these mutants exhibited a marked decrease in the activity, indicating that the sequence CCCC is important for the IL-1α promoter activation.

Sp1 activates the IL-1α promoter activity in a manner dependent on the GC box in A375 cells

It is known that Sp1 and Sp3 bind to the GC box in the promoter region of many genes. To investigate the effect of Sp1 on the IL-1α promoter activity, we transfected A375 cells with an expression plasmid encoding Sp1, together with IL-1α reporter plasmids, and determined the luciferase activity. As shown in **Figure 2A**, Sp1 markedly augmented the luciferase activity of pGL3-IL-1a(−51) and pGL3-IL-1a(−70) in A375-R8 cells. A small increase in the transcription activity was observed in pGL3-IL-1a(−31), suggesting that the GC box or GC box-like element present in pGL3-IL-1a(−31) or the vector may be operating. However, the augmenting effect was not observed in the GC box mutant, pGL3-IL-1a(421)ΔCC or pGL3-IL-1a(421)mATAA. These results indicate that Sp1 activates IL-1α promoter activity in a manner dependent on the GC box. Next, we compared the effects of wild-type Sp1, mutant Sp1, and Sp3 on the IL-1α promoter activity in A375-6 and A375-R8 cells. As shown in Figure 2B, wild-type Sp1 markedly augmented the IL-1α promoter activity in A375-R8 cells and less effectively in A375-6 cells. In contrast, Sp1ΔN and Sp1ΔC, which lack the transactivation domain and the DNA binding domain, respectively [27], and wild-type Sp3 did not augment the promoter activity. These results suggest that Sp1 but not Sp3 activates the IL-1α promoter activity, and the transactivation domain and the DNA binding domain are necessary for Sp1 transactivation activity.