The interaction between monocytes and tumor cells promotes monocyte differentiation and tumor cell invasion

～Role of cell adhesion to extracellular matrix～

単球とがん細胞の相互作用は，単球分化とがん細胞浸潤を促進する

～細胞外マトリックスとの細胞接着の役割～

Go Kamoshida

鴨志田 剛

This dissertation is dedicated to my father and mother.

論 文 目 録

主論文

1) **Go Kamoshida**, Ayaka Matsuda, Wakana Sekine, Hiromi Mizuno, Teruaki Oku, Saotomo Itoh, Tatsuro Irimura and Tsutomu Tsuji
Monocyte differentiation induced by co-culture with tumor cells involves RGD-dependent cell adhesion to extracellular matrix
Cancer Letters, 315, 145-152 (2012).

2) **Go Kamoshida**, Ayaka Matsuda, Risa Miura, Yuri Takashima, Arisa Katsura and Tsutomu Tsuji
Potentiation of tumor cell invasion by co-culture with monocytes accompanying enhanced production of matrix metalloproteinase and fibronectin
Clinical & Experimental Metastasis, in press.

参考論文

1) Saotomo Itoh, Eri Hamada, **Go Kamoshida**, Ryosuke Yokoyama, Takemasa Takii, Kikuo Onozaki, and Tsutomu Tsuji
Staphylococcal superantigen-like protein 10 (SSL10) binds to human immunoglobulin G (IgG) and inhibits complement activation via the classical pathway
Molecular Immunology, 47, 932-938 (2010).

2) Saotomo Itoh, Eri Hamada, **Go Kamoshida**, Kana Takeshita, Teruaki Oku and Tsutomu Tsuji
Staphylococcal superantigen-like protein 5 inhibits matrix metalloproteinase 9 from human neutrophils
Infection and Immunity, 78, 3298-3305 (2010).

3) Ryosuke Yokoyama, Saotomo Itoh, **Go Kamoshida**, Takemasa Takii, Satoshi Fujii, Tsutomu Tsuji and Kikuo Onozaki
Staphylococcal superantigen-like protein 3 binds to the toll-like receptor 2 extracellular domain and inhibits cytokine production induced by staphylococcus aureus, cell wall component, or lipopeptides in murine macrophages
Infection and Immunity, 80, 2816-2825 (2012).

論 文 目 録

4) Junsuke Suzuki, Eri Hamada, Tomonori Shodai, **Go Kamoshida**, Sanae Kudo, Saotomo Itoh, Junzo Koike, Kisaburo Nagata, Tatsuro Irimura and Tsutomu Tsuji
Cytokine secration from human monocytes potentiated by P-selectin-mediated cell adhesion
International Archives of Allergy and Immunology, 160, 152-160 (2012).

5) **Go Kamoshida**, Ayaka Matsuda, Kouji Katabami, Takumi Kato, Hiromi Mizuno, Wakana Sekine, Teruaki Oku, Saotomo Itoh, Makoto Tsuiji, Yoshiyuki Hattori, Yoshie Maitani and Tsutomu Tsuji
Involvement of transcription factor Ets-1 in the expression of the α3 integrin subunit gene
FEBS Journal, 279, 4535-4546 (2012).

発 表 目 録

1) 鴨志田剛, 松田彩花, 辻勉
がん細胞との共培養によるヒト単球の機能変化 ～マトリックスメタロプロテイナーゼの産生誘導～
第 10 回 Pharmaco-Hematology シンポジウム 2009 年

2) 伊藤佐生智, 濱田恵里, 鴨志田剛, 竹下佳奈, 瀧井猛将, 小野嵜菊夫, 奥輝明, 辻勉
黄色ブドウ球菌スーパー抗原様タンパク質 SSL5 による好中球マトリックスメタロプロテイナーゼの阻害
第 10 回 Pharmaco-Hematology シンポジウム 2009 年

3) Saotomo Itoh, Ryosuke Yokoyama, Eri Hamada, Go Kamoshida, Kana Takeshita, Teruaki Oku, Takemasa Takii, Kikuo Onozaki and Tsutomu Tsuji
Staphylococcal superantigen-like protein 5 (SSL5) inhibits matrix metalloproteinase-9 from human neutrophils
17th International Symposium on Mocelular Cell Biology of Macrophages 2009 年

4) 伊藤佐生智, 横山領介, 濱田恵里, 鴨志田剛, 竹下佳奈, 瀧井猛将, 小野嵜菊夫, 奥輝明, 辻勉
黄色ブドウ球菌スーパー抗原様タンパク質 SSL5 による好中球マトリックスメタロプロテイナーゼの阻害
第 21 回 微生物シンポジウム 2009 年

5) 鴨志田剛, 松田彩花, 辻勉
単球とがん細胞の相互作用: 単球からのマトリックスメタロプロテイナーゼ (MMP) 産生とがん細胞の浸潤
Interaction between monocytes and tumor cells: matrix metalloproteinase production and tumor cell invasion
第 82 回 日本生化学会大会 2009 年

6) 松田彩花, 鴨志田剛, 辻勉
がん細胞のアポトーシスに対する α3β1 インテグリン-ラミニン 5 媒介性接着の影響
Effect of alpha3beta1 integrin/laminin5-mediated adhesion on apoptosis of tumor cells
第 82 回 日本生化学会大会 2009 年

7) 伊藤佐生智, 濱田恵里, 鴨志田剛, 横山領介, 瀧井猛将, 小野嵜菊夫, 辻勉
黄色ブドウ球菌のスーパー抗原様タンパク質 SSL10 はヒト IgG に結合し古典経路による補体活性化を抑制する
日本薬学会 第 130 年会 2010 年

発 表 目 録

8) 鴨志田剛, 松田彩花, 辻勉
がん細胞との相互作用による単球の形質変化
第 11 回 Pharmaco-Hematology シンポジウム 2009 年

9) Go Kamoshida, Ayaka Matsuda, Tsutomu Tsuji
Phenotypic changes of human peripheral blood monocytes induced by co-culture with tumor cells: A role of extracellular matrix (ECM) proteins
14th International Congress of Immunology 2010 年

10) Saotomo Itoh, Eri Hamada, Go Kamoshida, Takemasa Takii, Kikuo Onozaki, Tsutomu Tsuji
Staphylococcal superantigen-like protein 10 binds to human immunoglobulin G and inhibits the classical complement activation pathway
14th International Congress of Immunology 2010 年

11) 横山領介, 伊藤佐生智, 鴨志田剛, 瀧井猛将, 辻勉, 小野嵜菊夫
Staphylococcal superantigen-like protein 6 (SSL6) はブタフィコリン β に結合する
第 56 回 日本薬学会東海支部総会・大会 2010 年

12) 伊藤佐生智, 濱田恵里, 鴨志田剛, 横山領介, 瀧井猛将, 小野嵜菊夫, 辻勉
黄色ブドウ球菌スーパー抗原様タンパク質 SSL10 はヒト IgG の Fc 部に結合し古典経路による補体活性化を抑制する
第 22 回 微生物シンポジウム 2010 年

13) 伊藤佐生智, 濱田恵里, 鴨志田剛, 横山領介, 瀧井猛将, 小野嵜菊夫, 辻勉
黄色ブドウ球菌スーパー抗原様分泌タンパク質 SSL ファミリーによる補体活性化抑制作用
フォーラム 2010 衛生薬学・環境トキシコロジー 2010 年

14) 鴨志田剛, 松田彩花, 辻勉
がん細胞との相互作用によるヒト末梢血単球の形質変化～細胞外マトリックス (ECM) タンパク質の役割～
Phenotypic changes of human peripheral blood monocytes induced by co-culture with tumor cells: A role of extracellular matrix (ECM) proteins
第 33 回 日本分子生物学会年会 第 83 回 日本生化学会大会 合同学会 2010 年

15) 横山領介, 伊藤佐生智, 鴨志田剛, 瀧井猛将, 辻勉, 小野嵜菊夫
黄色ブドウ球菌分泌タンパク質 SSL6 は ficolin-β に結合する
日本薬学会 第 131 回年会 2011 年

発 表 目 録

16) 鴨志田剛, 松田彩花, 桂有沙, 三浦莉紗, 高嶋友理, 辻勉
ヒト末梢血単球とがん細胞の相互作用 ～細胞外マトリックスタンパク質の働きとがん浸潤～
第 12 回 Pharmaco-Hematology シンポジウム 2011 年

17) 伊藤佐生智, 横山領介, 鴨志田剛, 岡田浩美, 藤井聡, 瀧井猛将, 小野嵜菊夫, 辻勉
黄色ブドウ球菌分泌タンパク質 SSL ファミリーの血漿中標的分子の探索
第 12 回 Pharmaco-Hematology シンポジウム 2011 年

18) 鴨志田剛, 辻勉
RGD 依存的細胞接着を介した単球の腫瘍馴化マクロファージへの分化とがん細胞浸潤への影響
第 20 回 日本がん転移学会学術集会・総会 2011 年

19) 伊藤佐生智, 横山領介, 鴨志田剛, 岡田浩美, 藤井聡, 瀧井猛将, 辻勉, 小野嵜菊夫
黄色ブドウ球菌スーパー抗原様タンパク質 SSL10 は血液凝固因子を標的とし, 血液凝固を抑制する
第 23 回 微生物シンポジウム 2011 年

20) 伊藤佐生智, 横山領介, 鴨志田剛, 岡田浩美, 藤井聡, 瀧井猛将, 辻勉, 小野嵜菊夫
黄色ブドウ球菌スーパー抗原様タンパク質 SSL10 による血液凝固抑制作用
フォーラム 2011 衛生薬学・環境トキシコロジー 2011 年

21) 鴨志田剛, 松田彩花, 桂有沙, 三浦莉紗, 高嶋友理, 辻勉
細胞外マトリックスタンパク質を介した単球とがん細胞の相互作用による形質変化
Phenotypic changes of monocytes and tumor cells induced by co-culture via extracellular matrix proteins
第 84 回 日本生化学会大会 2011 年

22) 伊藤佐生智, 横山領介, 鴨志田剛, 岡田浩美, 藤井聡, 瀧井猛将, 辻勉, 小野嵜菊夫
黄色ブドウ球菌スーパー抗原様タンパク質 SSL10 の血液凝固抑制作用
日本薬学会 第 132 年会 2012 年

23) 鴨志田剛, 三浦莉紗, 高嶋友理, 鈴木沙由里, 松田彩花, 辻勉
単球との相互作用によるがん細胞の形質変化 ～がん細胞浸潤とフィブロネクチン～
第 13 回 Pharmaco-Hematology シンポジウム 2012 年

24) 伊藤佐生智, 横山領介, 鴨志田剛, 村瀬千鶴子, 岡田浩美, 藤井聡, 瀧井猛将, 辻勉, 小野嵜菊夫
黄色ブドウ球菌分泌タンパク質 SSL10 は Gla ドメインを介して血液凝固因子に結合し, 血液凝固を抑制する
第 13 回 Pharmaco-Hematology シンポジウム 2012 年

発 表 目 録

25) 鴨志田剛, 松田彩花, 桂有沙, 高嶋友理, 三浦莉紗, 辻勉
転写因子 Ets-1 による細胞接着分子 α3 インテグリンの発現調節
平成 24 年度 日本生化学会関東支部例会 2012 年

26) 鴨志田剛, 辻勉
転写因子 Ets-1 による細胞接着分子 α3 インテグリンの発現調節
Regulation of alpha3 integrin gene expression by transcription factor Ets-1
第 21 回 日本がん転移学会学術集会・総会 2012 年

27) 横山領介, 伊藤佐生智, 鴨志田剛, 瀧井猛将, 藤井聡, 辻勉, 小野寄菊夫
黄色ブドウ球菌スーパー抗原様タンパク質 SSL3 は toll like receptor 2 に結合しマクロファージのサイトカイン産生を抑制する
第 24 回 微生物シンポジウム 2012 年

28) 横山領介, 伊藤佐生智, 鴨志田剛, 奥村拓也, 瀧井猛将, 藤井聡, 辻勉, 小野寄菊夫
黄色ブドウ球菌分泌毒素 Staphylococcal superantigen like 3 は toll like receptor 2 (TLR2) に結合し, TLR2 リガンドによるマクロファージからの炎症性サイトカイン産生を抑制する
フォーラム 2012 衛生薬学・環境トキシコロジーシンポジウム 2012 年

29) 鴨志田剛, 松田彩花, 三浦莉紗, 高嶋友理, 桂有沙, 辻勉
単球との共培養によるマトリックスメタロプロテイナーゼとフィブロネクチン産生亢進を介したがん細胞浸潤能増強
Potentiation of tumor cell invasion by co-culture with monocytes accompanying enhanced production of matrix metalloproteinase and fibronectin
第 85 回 日本生化学会大会 2012 年

30) 横山領介, 伊藤佐生智, 鴨志田剛, 奥村拓也, 瀧井猛将, 藤井聡, 辻勉, 小野寄菊夫
黄色ブドウ球菌スーパー抗原様タンパク質 SSL3 は toll like receptor 2 の細胞外領域に結合し, TLR2 を介したマクロファージからの炎症性サイトカイン産生を抑制する
日本薬学会 第 133 年会 2013 年

特 許

1) 特許出願 出願番号: 特願 2011-090473 発明の名称: 抗凝固薬およびその用途

The interaction between monocytes and tumor cells promotes monocyte differentiation and tumor cell invasion

～Role of cell adhesion to extracellular matrix～

単球とがん細胞の相互作用は，単球分化とがん細胞浸潤を促進する
～細胞外マトリックスとの細胞接着の役割～

D003 鴨志田 剛 (Kamoshida, Go)

がん組織において，腫瘍細胞と近傍の非腫瘍細胞および細胞外マトリックス (extracellular matrix: ECM) との相互作用によって作り出されるがん微小環境 (tumor microenvironment) は，がんの進行に重要であると考えられている．従って，がんを理解するためには，これらがんを取り巻く微小環境を理解することが必要不可欠であると考えられる．がん組織の間質に浸潤するマクロファージは腫瘍関連マクロファージ (tumor-associated macrophage: TAM) と呼ばれ，がん細胞の挙動に影響を与えている．TAM は抗腫瘍作用を発揮する場合もあるが，一方でがん浸潤・転移や血管新生を誘導し，腫瘍進展に対し促進的に働くこともあり，がん患者の予後を良好あるいは不良に導く二面的な作用が知られている．TAM は様々な種類のがん細胞との相互作用により多様な形質を示すことが考えられ，その性状の詳細については未解明である．

本研究ではまず，TAM の性質の理解，分化誘導の機序を解明するため，マクロファージ前駆細胞である単球をがん細胞と共培養し，*in vitro* で TAM 様細胞 (腫瘍馴化マクロファージ: Tumor-conditioned macrophage) の誘導を試みた．ヒト末梢血単球は，ヒトがん細胞株と 3-7 日間共培養することにより，マクロファージ様の伸展した形態を示し，マトリックスメタロプロテイナーゼ (MMP)-9 産生能をもつ腫瘍馴化マクロファージに形質が変化した．この形質変化誘導能は，ヒト胃がん細胞株 MKN1 とヒトグリオーマ細胞株 A172 では強く，ヒト腎臓がん細胞株 SN12C

とヒト膀胱がん細胞株 EJ-1 では弱かった．このことから，共培養に用いるがん細胞の種類により単球の形質変化の程度が異なることも示された．次に，がん細胞のマトリゲルへの浸潤に及ぼす影響を検討したところ，腫瘍馴化マクロファージの共存により促進され，この促進効果は，共存する腫瘍馴化マクロファージの MMP-9 産生能と相関していた．

次に，MMP-9 産生能と単球の形態変化との間に相関が認められたことから，単球分化における ECM タンパク質の役割について検討を行った．単球とがん細胞の共培養系中に，インテグリン媒介性の細胞接着に対し阻害作用をもつ RGD (Arg-Gly-Asp) ペプチドを添加することにより，単球の分化誘導が顕著に阻害された．さらに，がん細胞が産生するフィブロネクチンを gelatin-Sepharose で吸収することによっても単球の分化誘導が阻害された．また，フィブロネクチンをあらかじめコートしたプレートを用い共培養することによっても，単球の形質変化誘導が促進された．これらのことから，このような単球の形質変化に，がん細胞が産生するフィブロネクチンなどの ECM タンパク質が重要な役割を果たしていることが明らかとなった．

本研究から，単球の形質変化に RGD 依存的細胞接着が重要な役割を果たしていることが示された．また，このように分化した腫瘍馴化マクロファージは，MMP-9 産生を通じて，がんの浸潤・転移や血管新生に影響を与えることが示唆された．さらに，本研究で用いた腫瘍馴化マクロファージのモデルは，TAM の性質，分化誘導機序を明らかにするための有用なモデルであると思われる．

これまでに，単球はがん細胞との相互作用で，腫瘍馴化マクロファージへと形質を変えることを明らかにしたが，がん細胞も単球との相互作用により，形質を変化させている可能性を考え，がん細胞の浸潤能に注目し解析を行った．

腫瘍馴化マクロファージを誘導したモデルを応用し，ヒトがん細胞株とヒト末梢血単球を 5 日間共培養し，共培養後のがん細胞の形質を解析した．マトリゲルへの浸潤能および MMP-9 産生能を評価したところ，MKN1 および HT1080 (ヒト線維芽肉腫) 細胞では，単球との共培養により，浸潤能および MMP-9 産生能の増強が認められた．しかし，EJ-1, A172, SN12C 細胞では，そのような形質変化は認められなかった．形質変化を詳しく解析したところ，MKN1 細胞は単球との 5 日間の共培養に

より，非常に伸展した形態を示し，間葉系マーカーであるビメンチンの発現が上昇した．また，フィブロネクチン産生およびその受容体である α5β1 インテグリン発現も単球との共培養により増加することが示された．さらに，MKN1 細胞の浸潤能亢進は RGD ペプチドにより抑制され，単球との共培養により，細胞接着で活性化されるシグナルカスケードの 1 つである focal adhesion kinase (FAK) のリン酸化も認められた．HT1080 細胞でも単球との 5 日間の共培養により，MKN1 細胞同様フィブロネクチンおよび α5β1 インテグリン発現の増強が認められた．しかし，単球との共培養を行っても浸潤能および MMP-9 産生能の増強が認められなかったがん細胞株では，フィブロネクチンおよび α5β1 インテグリン発現の増強も起こらなかった．これらのことから，一部のがん細胞では，単球との相互作用で誘導される浸潤能および MMP-9 産生能の亢進に，フィブロネクチン-α5β1 インテグリン相互作用が関与していることが示唆された．

次に，がん細胞の形質変化誘導に関わる因子について解析を試みた．近年，tumor necrosis factor (TNF)-α などの炎症性サイトカインが，がんの悪性挙動を促進することが報告されている．単球/マクロファージはしばしば，TNF-α を産生することが知られている．そこで，単球の培養上清を MKN1 細胞に作用させたところ，浸潤能の亢進が認められたが，培養上清を抗 TNF-α 抗体および protein G-Sepharose で処理し，TNF-α を吸収することによって MKN1 細胞の浸潤能亢進が抑制された．また，MKN1 細胞に TNF-α を直接作用させることによっても，浸潤能，MMP-9 産生能およびフィブロネクチン産生能が亢進した．これらのことから，がん細胞の形質変化の一部には，単球が産生する TNF-α が関与することが示唆された．

がん細胞も単球との相互作用により，その形質を変化させ，高い浸潤・転移能，血管新生能などを獲得し，がん進展に促進的に働くことが推察された．さらに，がん細胞からのフィブロネクチン産生が増強することから，単球の TAM への分化にも促進的に作用し，がん細胞と単球の形質変化が相乗的にがんを進展させることが示唆された．

本研究から，単球とがん細胞の相互作用によるそれぞれの細胞の形質変化には，フィブロネクチンなどの ECM タンパク質を介した細胞接着が重要な役割を果たすことが明らかとなった．がん微小環境における

ECM タンパク質を介する細胞接着の役割を理解することは，がんの悪性化機序の解明へと繋がり，がんの予防や治療に貢献するものと考えられる．

Table of contents

List of publications

1) **Go Kamoshida**, Ayaka Matsuda, Wakana Sekine, Hiromi Mizuno, Teruaki Oku, Saotomo Itoh, Tatsuro Irimura and Tsutomu Tsuji
Monocyte differentiation induced by co-culture with tumor cells involves RGD-dependent cell adhesion to extracellular matrix
Cancer Letters, 315, 145-152 (2012).

2) **Go Kamoshida**, Ayaka Matsuda, Risa Miura, Yuri Takashima, Arisa Katsura and Tsutomu Tsuji
Potentiation of tumor cell invasion by co-culture with monocytes accompanying enhanced production of matrix metalloproteinase and fibronectin
Clinical & Experimental Metastasis, in press

Abbreviations

本論文では，以下の略語を用いた.

ECM; extracellular matrix, 細胞外マトリックス

FCS; fetal calf serum, ウシ血清

IL; interleukin, インターロイキン

M-CSF; macrophage colony-stimulating factor, マクロファージコロニー刺激因子

MMP; matrix metalloproteinase, マトリックスメタロプロテアーゼ

RGD; arginyl-glycyl-aspartic acid, アルギニン-グリシン-アスパラギン酸

RT-PCR; reverse transcription-polymerase chain reaction, 逆転写ポリメラーゼ連鎖反応

TAM; tumor-associated macrophage, 腫瘍関連マクロファージ

TNF-α; tumor necrosis factor-α, 腫瘍壊死因子-α

General Introduction

Cancer is the leading cause of death in Japan, and approximately one-third of the population dies from cancer. Although recent progress in cancer therapy has improved patients' prognosis, metastasis is still a big problem. Metastasis is a complex multi-step process that has not yet fully understood. Recently, the notion that tumor microenvironment composed of tumor cells, host cells and extracellular matrix (ECM) affects behaviors of tumor cells and host cells has been widely accepted. The mutual interactions between tumor cells and cellular and non-cellular host components regulate tumor development and progression by controlling proliferation, migration, invasion, metastasis, angiogenesis, and apoptosis [1-3]. Thus, it is important to investigate tumor microenvironment for better understanding of malignant behavior of tumor cells.

Recent interest in tumor-stroma interactions associated with tumor progression has been focused on how stromal cells contribute to the changes of malignant phenotypes of tumor cells. It has been reported that endothelial cells, fibroblasts and various cells derived from bone marrow are infiltrating into tumor tissues [4]. Among them, macrophages are a major population of host immune cells that infiltrate into tumor tissues, and such macrophages are referred to as tumor-associated macrophages (TAMs) (Chart 1). Although macrophages have been thought to exhibit anti-tumor activity and exert protective functions against tumor development, recent studies have demonstrated that TAMs promote the proliferation, invasion, and metastasis of tumor cells and angiogenesis in tumor tissues [5-7]. Indeed, TAMs have been reported to secrete various cytokines, growth factors, and enzymes promoting tumor metastasis and angiogenesis, including vascular endothelial growth factor (VEGF) [8], interleukin (IL)-8 [9], epidermal growth factor (EGF) [10], platelet-derived growth factor (PDGF) [11], tumor necrosis factor (TNF)-α [12,13], and matrix metalloproteinases (MMPs) [13,14]. The infiltration of TAMs into solid tumors, especially in breast, ovarian, prostate, and cervical cancer, is frequently correlated with poor prognosis [15-17]. TAMs are derived from circulating monocytes that are recruited into tumor tissues by chemokines and cytokines such as monocyte chemotactic protein (MCP)-1 [18] and VEGF [19] produced by tumor cells. These monocytes are then differentiated to TAMs in the tumor tissues. However, little is known about the process in which monocytes are differentiated to TAMs in tumor tissues. Thus, it seems to be

important to clarify how circulating monocytes are differentiated to TAMs and what characteristics TAMs acquire by their interaction with tumors.

Macrophages are shown to be classified into two major subsets; i.e. M1, a classic immunoactive macrophages and M2, tissue remodeling macrophages by stimulation from microenvironment [20]. It has been postulated that TAMs exhibit immunosuppressive nature with phenotypes similar to those of M2-type macrophages, but functions of TAMs in tumor invasion and metastasis have not been sufficiently characterized [21]. In addition, TAMs seem to be heterogeneous in their biological nature because "TAM" is designated simply based on its location in tumor tissues. It is possible that monocytes recruited to the tumor lesion are differently influenced by the interactions with neighboring tumor cells.

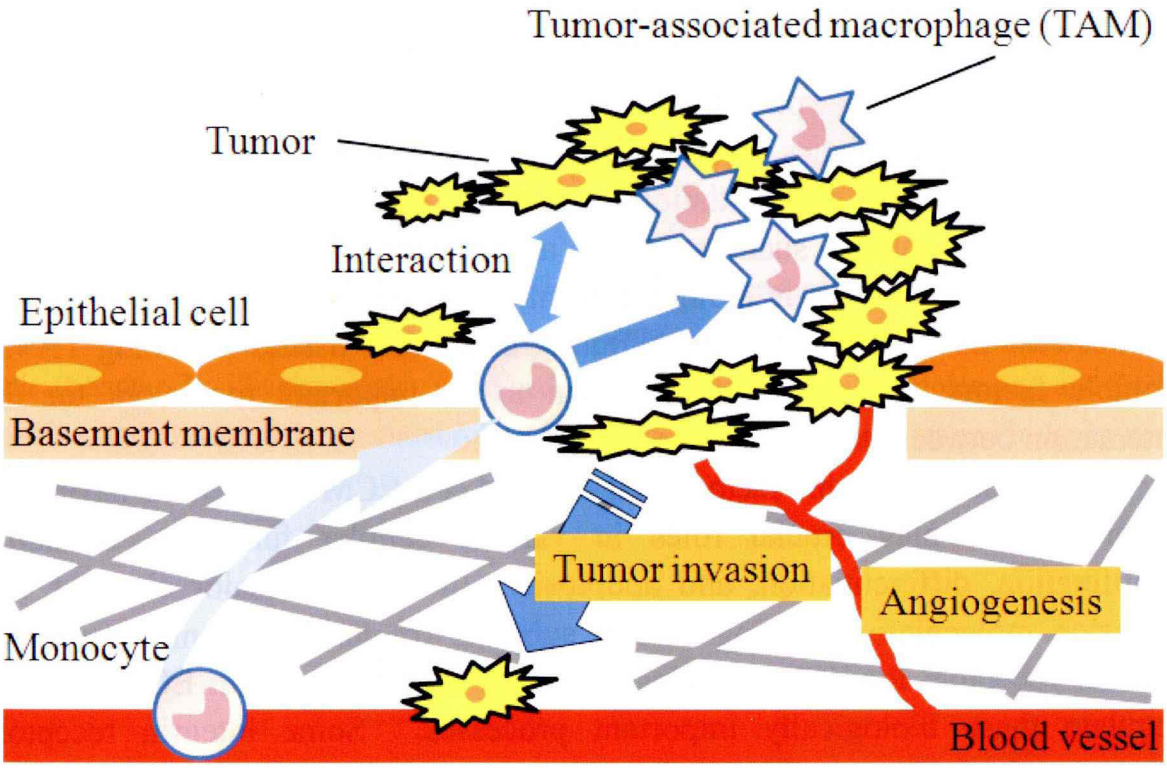

Chart 1. Schematic view of the interaction of tumor cells and tumor-associated macrophages (TAMs). Monocytes recruited into tumor tissues interact with tumor cells and differentiate to TAMs. The malignant behaviors of tumor cells such as invasion and angiogenesis are greatly influenced by the interaction with TAMs.

Several studies have been carried out to characterize TAMs mainly using mouse *in vivo* models or clinical specimens. Because, these cells have already been differentiated to macrophages, there is difficulty in the isolation of infiltrating macrophages without loss of activity from solid tumors. Therefore, I attempted to establish *in vitro* experiment system to induce TAMs from human peripheral blood monocytes by co-culture with various human tumor cell lines. We analyzed matrix metalloproteinase (MMP)-9 as a differentiation marker for macrophages because MMP-9 was considered to be one of common features of TAMs and involved in tumor invasion, metastasis, and angiogenesis [13,14,22-24].

Macrophage colony-stimulating factor (M-CSF) has been considered as one of the most important cytokines that induce TAM generation [24,25]. In addition, autocrine production of immunosuppressive cytokine IL-10 was reported to mediate the differentiation of TAMs [26]. It was recently reported that leukemia inhibitory factor (LIF), IL-6 [27], and TNF-α [13,23] contributed to the differentiation of TAMs. Transcriptional regulator NF-κB-dependent signaling has also been shown to play an important role in the generation and maintenance of immunosuppressive phenotypes of TAMs [28,29]. Thus, various factors including these cytokines, were thought to cooperatively participate in the process of TAM's differentiation. Recent studies have indicated that inflammatory cytokines such as TNF-α accelerate the malignant behaviors of tumor cells [12,13,30,31]. It is thought that most of these inflammatory cytokines are secreted from immune cells infiltrating into tumor tissues including TAMs. Soluble factors such as cytokines seem to be important mediators for the interaction between tumor cells and monocytes.

In addition to soluble factors, cell-cell and cell-ECM interactions have been considered to play crucial roles in various cellular functions, including proliferation, differentiation, and apoptosis. Integrins, a major class of cell adhesion molecules consisting of two subunits (α and β subunits), serve as cellular receptors for ECM proteins, and their interactions with ECM proteins regulate these biologically important processes. Some integrin receptors recognize the RGD (Arg-Gly-Asp) sequence present in adhesive ECM proteins such as fibronectin and laminin [32,33]. The interactions of integrin receptors with ECM proteins transduce signals to modulate cellular functions. In the present study, I evaluated contribution of ECM proteins to the differentiation/activation mechanisms of TAMs and potentiation of malignant behaviors of tumor cells. I investigated how the interactions between monocytes and tumor cell lines change phenotypes of these cells, focusing on cell adhesion to ECM proteins. In Chapter 1, I attempted to induce monocytes

to differentiate into TAM-like cells by co-culture with tumor cells, and evaluated the potential role of cell adhesion to ECM proteins in this process. In Chapter 2, I focused on phenotypic changes of tumor cells after the co-culture with monocytes, and assessed the roles of inflammatory cytokines such as TNF-α produced by monocytes (Chart 2).

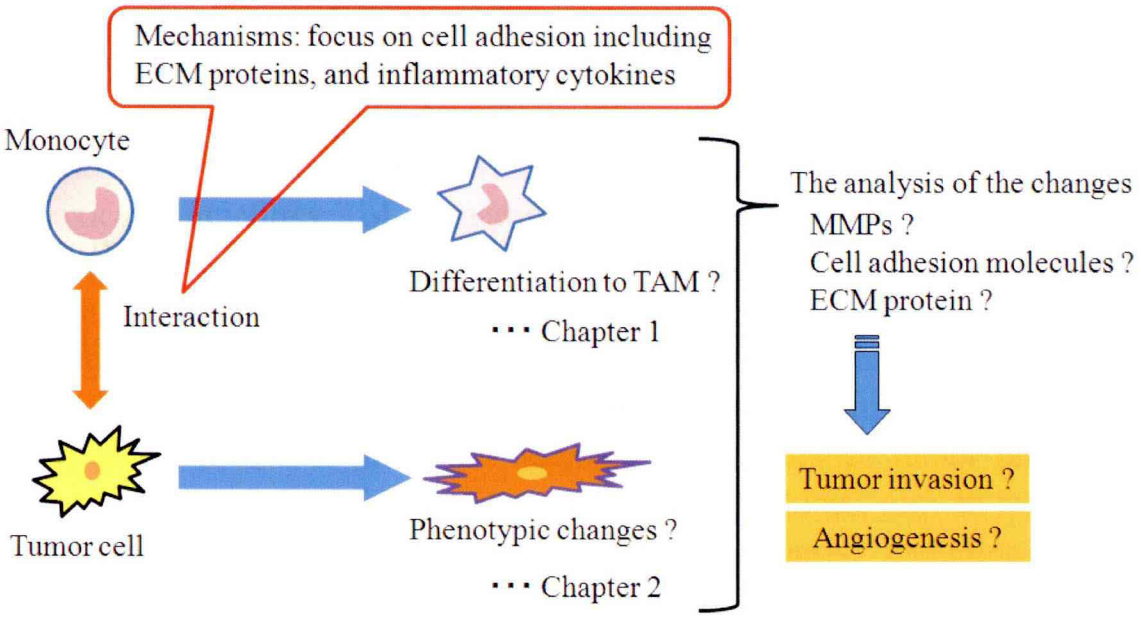

Chart 2. Schematic view of this study. The interaction between monocytes and tumor cells induces differentiation of monocytes to TAMs (Chapter 1) and phenotypic changes of tumor cells (Chapter 2). In this study, I focused on MMPs, cell adhesion molecules, and ECM proteins, and evaluated the potential roles of cell adhesion to ECM proteins and inflammatory cytokines in these processes.

Chapter 1

Monocyte differentiation induced by co-culture with tumor cells involves RGD-dependent cell adhesion to extracellular matrix

Introduction

The malignant behavior of tumor cells is greatly influenced by the microenvironment. Macrophages are a major population of immune cells that infiltrate into tumor tissues; such macrophages are referred to as tumor-associated macrophages (TAMs). It was originally thought that immune cells, including macrophages, exhibit anti-tumor activity and exert protective functions against tumor development. However, the infiltration of TAM into solid tumors, especially in breast, ovarian, prostate and cervical cancers, frequently has been correlated with poor prognosis [15-17]. These apparently paradoxical functions of macrophages have been ascribed to the immunosuppressive nature of TAM with phenotypes similar to that of M2-type macrophages involved in tissue remodeling [21,24,26,34]. Recent studies have demonstrated that TAMs promote not only the proliferation, invasion, and metastasis of tumor cells, but also angiogenesis, which is supportive of growing tumors, and thus TAMs have also been shown to enhance tumor progression [5-7,35]. TAMs are indeed found to secrete various cytokines, growth factors, and enzymes promoting tumor metastasis and angiogenesis, including vascular endothelial growth factor (VEGF) [8], interleukin (IL)-8 [9], epidermal growth factor (EGF) [10], platelet-derived growth factor (PDGF) [11], tumor necrosis factor (TNF)-α [12,13], and matrix metalloproteinases (MMPs) [13,14].

TAMs are derived from circulating monocytes that are recruited into tumor tissues by chemokines such as monocyte chemotactic protein-1 (MCP-1) that are produced by tumor cells [18]. Macrophage colony-stimulating factor (M-CSF) has also been considered as one of the most important cytokines that induce TAM generation [24,25]. In addition, autocrine production of immunosuppressive cytokine IL-10 was reported to mediate the differentiation of TAMs [26]. It was recently reported that leukemia inhibitory factor (LIF), IL-6 [27], and TNF-α [13,23] contribute to the differentiation of TAMs. Transcriptional regulator NF-κB-dependent signaling has also been shown to play an important role in the generation and maintenance of the immunosuppressive phenotypes of TAMs [28,29]. Thus, various factors, including these cytokines, are thought to cooperatively participate in the process of TAM differentiation. Recently, Solinas and co-workers [24] successfully generated TAM-like cells by culturing monocytes with conditioned media of certain tumor cell lines. Their gene profiling analysis revealed that monocyte-derived cells have a gene expression pattern similar to that of TAMs isolated from human tumors, and they

designated these monocyte-derived cells as "tumor-conditioned macrophages". Moreover, the generation of such tumor-conditioned macrophages was suppressed by the depletion of M-CSF from conditioned media, and thus this system confirmed the central role played by M-CSF in the differentiation from monocytes to TAM-like cells. In addition to those played by soluble cytokines, significant roles in the differentiation of monocytes to macrophages were suggested for cell-to-matrix interactions [36,37]. To address the specific mechanisms involved in the initial phase of monocyte differentiation, we attempted to induce monocytes to differentiate, *in vitro*, into TAM-like cells by co-culture with tumor cells, and we evaluated the potential role of cell adhesion to extracellular matrix (ECM) proteins in this process.

In this study, we found that monocytes were induced to differentiate into macrophage-like cells, thereby producing high levels of MMP-9 (also called gelatinase B), the expression of which is a known characteristic of TAMs [7,13,23,35]. We also report the significance of cell adhesion in monocytic differentiation. Cell adhesion has recently been considered to play crucial roles in various cellular functions, including proliferation, differentiation, and apoptosis. Integrin is a major family of cell adhesion molecules consisting of two subunits (α and β subunits), and the combination of these subunits determines ligand specificity. Some integrin receptors recognize the RGD (Arg-Gly-Asp) sequence present in adhesive extracellular matrix (ECM) proteins such as fibronectin and laminin. We present evidence that RGD-dependent cell adhesion is implicated in monocyte differentiation into TAM-like cells.

Materials and methods

Reagents and antibodies

Heparin was purchased from Novo Nordisk (Copenhagen, Denmark). Dextran 200,000 and Ficoll-Paque™ PLUS were obtained from Wako Chemicals Industries (Osaka, Japan) and GE Healthcare (Buckinghamshire, UK), respectively. Matrigel™ and laminin-1 were purchased from BD Biosciences (San Diego, CA). Fibronectin was purchased from Chemicon International (Temecula, CA). Ex Taq polymerase was obtained from TaKaRa (Osaka, Japan). Trizol™, Super Script™ II reverse transcriptase, and CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) were products of Invitrogen (Carlsbad, CA). Inhibitor of MMP-9 (anthranilic acid derivative), inhibitor of MMP-2 (oleoyl-*N*-hydroxylamide), H-Gly-Arg-Gly-Asp-Ser-Pro-OH (GRGDSP) peptide, and H-Gly-Arg-Ala-Asp-Ser-Pro-OH (GRADSP) peptide were purchased from Calbiochem (San Diego, CA). Gelatin-Sepharose was a product of GE Healthcare.

Antibodies directed to MMP-9 and CD68 were purchased from R&D Systems (Minneapolis, MN). Antibodies against Mac-1 (αMβ2 integrin), CD14, and fibronectin were purchased from Nichirei Bioscience (Tokyo, Japan), Biolegend (San Diago, CA), and TaKaRa, respectively. Alexa Fluor 488-conjugated donkey anti-goat IgG antibody and Alexa Fluor 647-conjugated goat anti-mouse IgG antibody were purchased from Invitrogen. HRP-conjugated horse anti-goat IgG antibody and HRP-conjugated goat anti-mouse IgG antibody were purchased from Kirkegaard & Perry Laboratories (KPL; Gaithersburg, MD).

Cells

MKN1 (human gastric carcinoma cell line) was provided by the RIKEN Cell Bank (Tsukuba, Japan). A172 (a human glioma cell line) and EJ-1 (a human bladder carcinoma cell line) were provided by the Human Science Research Resources Bank (Osaka, Japan). SN12C (a human renal carcinoma cell line) was provided by Dr. Isaiah J. Fidler of the M.D. Anderson Cancer Center. The cells were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS) at 37°C under a 5% CO_2 atmosphere.

Monocytes were isolated from human peripheral blood essentially as described previously [38]. Briefly, heparinized human blood was mixed with an

equal volume of 3% dextran 200,000/saline to sediment most of the erythrocytes. After the samples were left to stand for 30 min, the supernatant was centrifuged at 1,200 rpm for 10 min. Pelleted cells were then subjected to Ficoll-Paque density gradient centrifugation at 1,200 rpm for 30 min, and mononuclear cells (PBMCs) were recovered. The PBMCs thus obtained were allowed to adhere to the culture dish in RPMI 1640/10% FCS at 37°C for 1 h, and adherent cells were collected by a cell scraper. The purity of monocytes was estimated to be 60-80% by flow cytometry (forward scattering/side scattering plots).

Co-culture of monocytes with tumor cells

Monocytes (1-2 x 10^5 cells) were added to a 24-well plate in which tumor cells had been cultured at a subconfluent density, and the co-culture was continued for 3-7 days in RPMI 1640/10% FCS at 37°C. In some experiments, the co-culture was conducted in a Boyden chamber system (Falcon 3504, 24-well culture plate; Falcon 3104, cell culture insert with PET membrane (pore size, 1.0 μm)) to inhibit direct cell-to-cell contact between monocytes and tumor cells.

Gelatin zymography

The MMP activity was detected by gelatin zymography essentially as described previously [39,40]. The culture supernatant was electrophoresed in polyacrylamide gel (6.5%) containing SDS (0.1%) and gelatin (1.5 mg/ml) under nonreducing conditions. The gel was washed three times in washing buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, pH 7.5) containing 2.5% Triton X-100 for 30 min, and then the gel was incubated in washing buffer lacking Triton X-100 at 37°C for 16 h. The gel was then stained with Coomassie brilliant blue (CBB) and destained with 7.5% acetic acid/5% methanol.

Immunoblotting

Specimens were subjected to SDS-polyacrylamide gel (7.5%) electrophoresis, and separated proteins were electrically blotted onto a nitrocellulose membrane (Hybond-ECL, GE Healthcare) using a semi-dry blotting system (BE-330, Bio-Craft, Tokyo, Japan). After the blotted membrane was blocked with 4% BlockAce™ (a milk protein-based blocking reagent; Dainippon Pharmaceutical, Osaka, Japan) for 30 min, the membrane was treated with anti-MMP-9 antibody (1:1000 dilution) for 20 h and then with HRP-conjugated horse anti-goat IgG antibody (1:1000 dilution) for 40 min.

The membrane was washed with PBS containing 0.05% Tween 20 (PBS-T) three times after each reaction step. Proteins were detected with an enhanced chemiluminescence (ECL) detection system (GE Healthcare).

For the detection of fibronectin in the culture supernatant, immunoblotting was also carried out, essentially under the same conditions as those described above. The sample was separated by SDS-polyacrylamide gel (6.5%) electrophresis, and the blotted membrane was treated with anti-fibronectin antibody (1:1000 dilution) for 20 h and HRP-conjugated goat anti-mouse IgG antibody (1:1000 dilution) for 40 min.

Immunofluorescence microscopy

Monocytes co-cultured with MKN1 cells in a Lab-Tek II chamber slide™ system (8-well glass slide; Nalge Nunc International 154534, Penfield, NY) were fixed with 1% formaldehyde at room temperature for 10 min and were treated with 0.2% Triton X-100/0.02% NaN_3 in PBS at 4°C for 10 min. The permeabilized cells were successively treated with 4% BlockAce™ for 30 min, anti-MMP-9 antibody (5 μg/ml) for 40 min, and anti-Mac-1 antibody (5 μg/ml) for 40 min. The cells were then stained with secondary antibodies (Alexa Fluor 488-conjugated donkey anti-goat IgG antibody (1:200 dilution) and Alexa Fluor 647-conjugated goat anti-mouse IgG antibody (1:200 dilution) for 30 min. After each reaction step, the cells were washed five times with PBS. The fluorescently labeled cells were observed with a confocal laser-scanning microscope (Radians 2100; Bio-Rad Laboratories, Hercules, CA).

Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was prepared by the AGPC method [41] using Trizol and was reverse-transcribed with Super Script II reverse transcriptase using an oligo-dT primer. In order to detect MMP-9 and β-Actin mRNA, the PCR was conducted with the following sets of primers: 5'-CCA TTT CGA CGA TGA CGA GTT-3' and 5'-CTT GTC GCT GTC AAA GTT CGA-3' for MMP-9 (GenBank accession number: NM 004994); and 5'-AAG ATG ACC CAG ATC ATG TTG AG-3' and 5'-AGG AGG AGC AAT GAT CTT GAT CTT-3' for β-actin (GenBank accession number: NM 001101). The conditions for the PCR were as follows: 95°C, 30 sec; 60°C, 30 sec; 72°C, 40 sec; 29-38 cycles. The products were separated in 2.0% agarose gel in 40 mM of Tris/acetate buffer containing 1 mM EDTA (pH 8.0). These procedures yield 544- and 648-bp products from the mRNAs for MMP-9 and β-actin, respectively.

Depletion of fibronectin from tumor cell-conditioned medium

The serum-free conditioned medium of MKN1 cells (1 ml) was mixed with gelatin-Sepharose (0.05 ml, 50% suspension), and the mixture was incubated at 4°C for 24 h. After the sedimentation of the gelatin-Sepharose, the supernatant was subjected to the immunoblotting analysis using anti-fibronectin antibody. Fibronectin was found to be effectively removed from the conditioned medium throughout the procedure. Proteins bound to the gelatin-Sepharose were analyzed by SDS-polyacrylamide gel electrophoresis followed by CBB staining, which yielded a major band of ~200 kDa that was identified as a subunit of fibronectin by immunoblot analysis.

In vitro invasion assay

The *in vitro* invasion assay was performed using a Boyden chamber system (Falcon 3504, 24-well culture plate; Falcon 3097, cell culture insert with PET membrane (pore size: 8.0 μm)), as described previously [42]. Monocytes (1 x 10^5 cells) that had been co-cultured with tumor cells in the Boyden chamber system were mixed with fluorescently labeled (1 μM CellTracker™ at 37°C for 30 min) MKN1 cells (1.25 x 10^5 cells), and the cell mixture was suspended in RPMI1640 (serum-free) and placed in the upper chamber of the Boyden system. The membrane between the upper and lower chambers had previously been coated with Matrigel™ at 4°C for 16 h. The lower chamber was filled with RPMI 1640/10% FCS, and the chambers were incubated at 37°C for 18 h. MKN1 cells that had migrated through Matrigel-coated membranes were counted under a fluorescence microscope. In some experiments, the cell invasion was assayed in the presence of an MMP-9 inhibitor (anthranilic acid derivative, 1 μM) or an MMP-2 inhibitor (oleoyl-*N*-hydroxylamide, 1 μM).

Flow cytometric analysis

The expression of CD14 and CD68 was measured with a flow cytometer (FACS Calibur, BD Biosciences, San Diego, CA) using specific antibodies and FITC-labeled anti-mouse IgG antibody, as described previously [43].

Results

Increases in MMP secretion by mixed culture of monocytes and tumor cells

Monocytes were co-cultured with MKN1, A172, SN12C, or EJ-1 cells for 3-7 days in RPMI 1640/10% FCS, and the culture media were replaced with serum-free RPMI 1640. After the cells had been cultured in serum-free medium for 24 h, the conditioned media were assayed for MMPs by gelatin zymography, and MMP-9 levels in the supernatants of the co-culture were found to be much higher than those of the single cultures of either monocytes or tumor cells alone (Fig. 1-1A). The increased secretion of MMP-9 after the co-culture of these cells for 3-7 days was observed in all four cases using distinct tumor cell lines, but the augmentation of MMP-9 secretion with MKN1 or A172 cells was more prominent than that with SN12C or EJ-1 cells. Although secretion of MMP-2 from MKN1 and A172 cells was detected, the levels of secretion remained unchanged by the presence of monocytes in the culture. We then performed an immunoblot analysis of the same culture supernatants using anti-MMP-9 antibody, and found that MMP-9 production in the co-culture supernatant had increased in a time-dependent manner (Fig. 1-1B). Because each sample used for zymography was collected 24 h after the replacement of old medium with fresh medium, the results suggest that the rate of MMP-9 secretion had gradually increased during this culture period. Taken together, these results indicate that MMP-9 secretion into culture media is potentiated by interactions between monocytes and tumor cells, and that the degree of this potentiation depends on the type of tumor cells present in the co-culture.

To address the role(s) played by direct cell-to-cell contact in the potentiation of MMP-9 production, we employed a Boyden chamber system for the co-culture. Co-culture in the Boyden chamber system still yielded high levels of MMP-9 secretion in all cases, i.e., with all four tumor cell lines used (Fig. 1-1C); however, MMP-9 levels in the Boyden chamber cultures were significantly lower than those of the regular mixed cultures. It is likely that in addition to soluble factors present in the culture, direct cell-to-cell interactions between monocytes and tumor cells played a significant role in the potentiation of MMP-9 secretion from these cells.

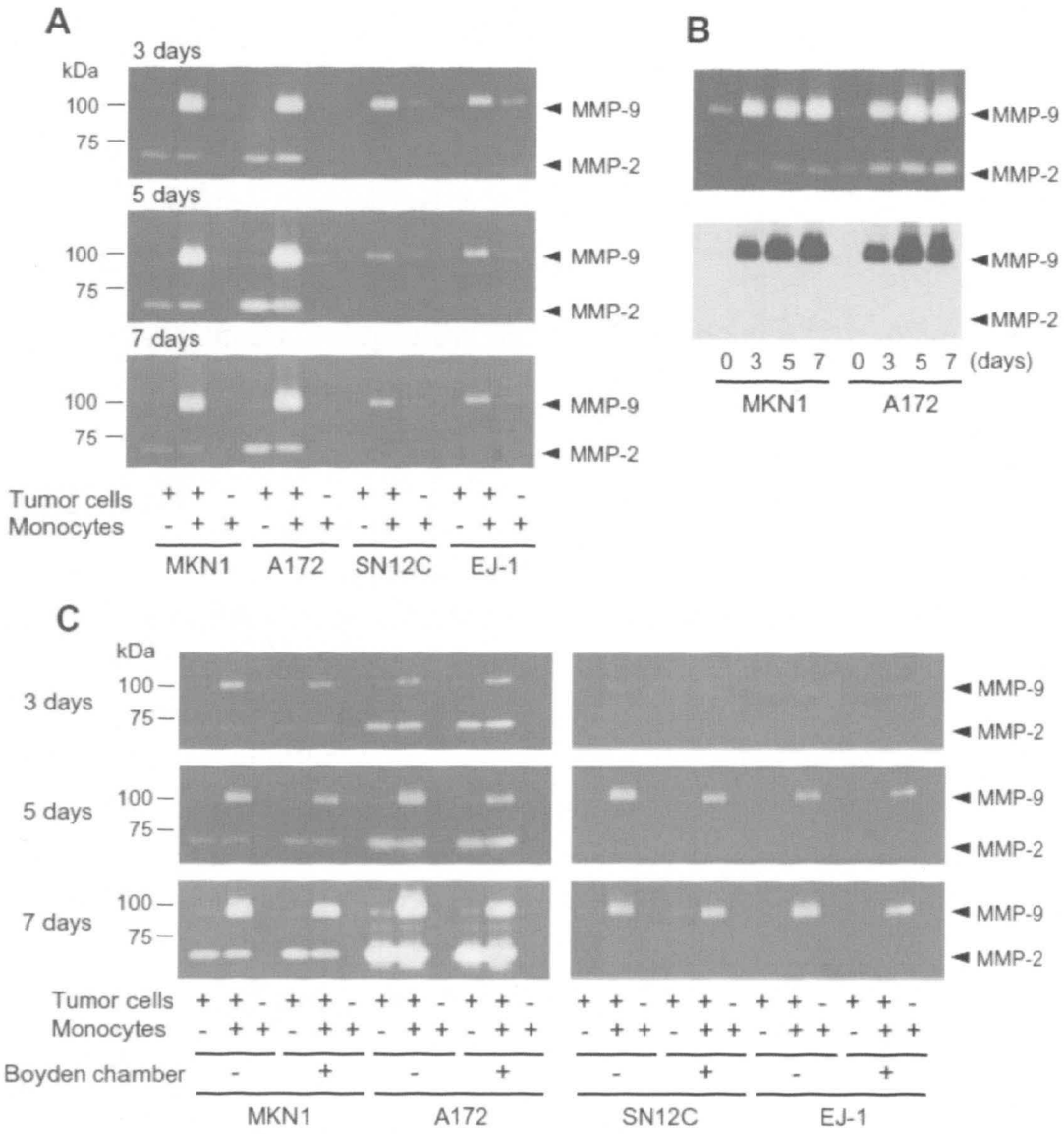

Fig. 1-1 Zymographic analysis of co-culture supernatants of monocytes and tumor cells. (A) Monocytes (1-2 x 10^5 cells) were cultured with subconfluent monolayers of MKN1, A172, SN12C, or EJ-1 cells for 3-7 days. The culture media were then replaced with fresh RPMI 1640 (serum-free), and the co-cultures were continued for 24 h. The supernatants were collected and assayed for MMPs by gelatin zymography. The secretion of MMPs from tumor cells or monocytes alone was also analyzed. (B) Serum-free conditioned media for 24 h after co-culture for 0-7 days, assayed by gelatin zymography (*upper panel*). The same samples were concentrated with Microcon (YM-30) and analyzed by immunoblotting using anti-MMP-9 antibody (*lower panel*). (C) Co-culture conducted with a Boyden chamber system. Tumor cells (2 x 10^5 cells) were placed in a 24-well culture plate (Falcon 3504) and cultured in RPMI 1640/10% FCS for 24 h. The medium was replaced with fresh serum-free RPMI 1640 (0.7 ml), and the plate was assembled with an upper chamber (Falcon 3104) containing a monocyte suspension (1-2 x 10^5 cells/0.3 ml). The chambers were incubated at

37°C for 3-7 days, and MMP in the culture supernatants was analyzed by gelatin zymography. Each conditioned medium from the co-culture with the Boyden chamber system (+) was compared to medium cultured on a regular 24-well plate (-).

We next performed immunofluorescence microscopy to determine which cells had been stimulated to produce MMP-9. Monocytes and MKN1 cells were co-cultured for 5 days, and the cells were stained with anti-MMP-9 and anti-Mac-1 antibodies after being permeabilized with 0.2% Triton X-100 at 4°C for 10 min (Fig. 1-2A). The strong fluorescence derived from anti-MMP-9 antibody was found to be associated with spherical cells rather than with flatten cells and the spherical cells were fluorescently positive for Mac-1, a monocytic surface marker. The finding suggests that monocytes produced MMP-9 more actively than did the MKN-1 cells. We also conducted an RT-PCR analysis of mRNA for MMP-9 in monocytes and MKN1 cells. After these two cell types were co-cultured for 5 days in the Boyden chamber system, the total RNA was isolated from both cell types, and was subjected to RT-PCR (Fig. 1-2B). The MMP-9 mRNA expression in monocytes increased after the co-culture with MKN1 cells, whereas that in the MKN1 cells did not markedly changed after co-culture with monocytes. Thus, it is most likely that the enhanced production of MMP-9 after co-culture was primarily of monocytic origin.

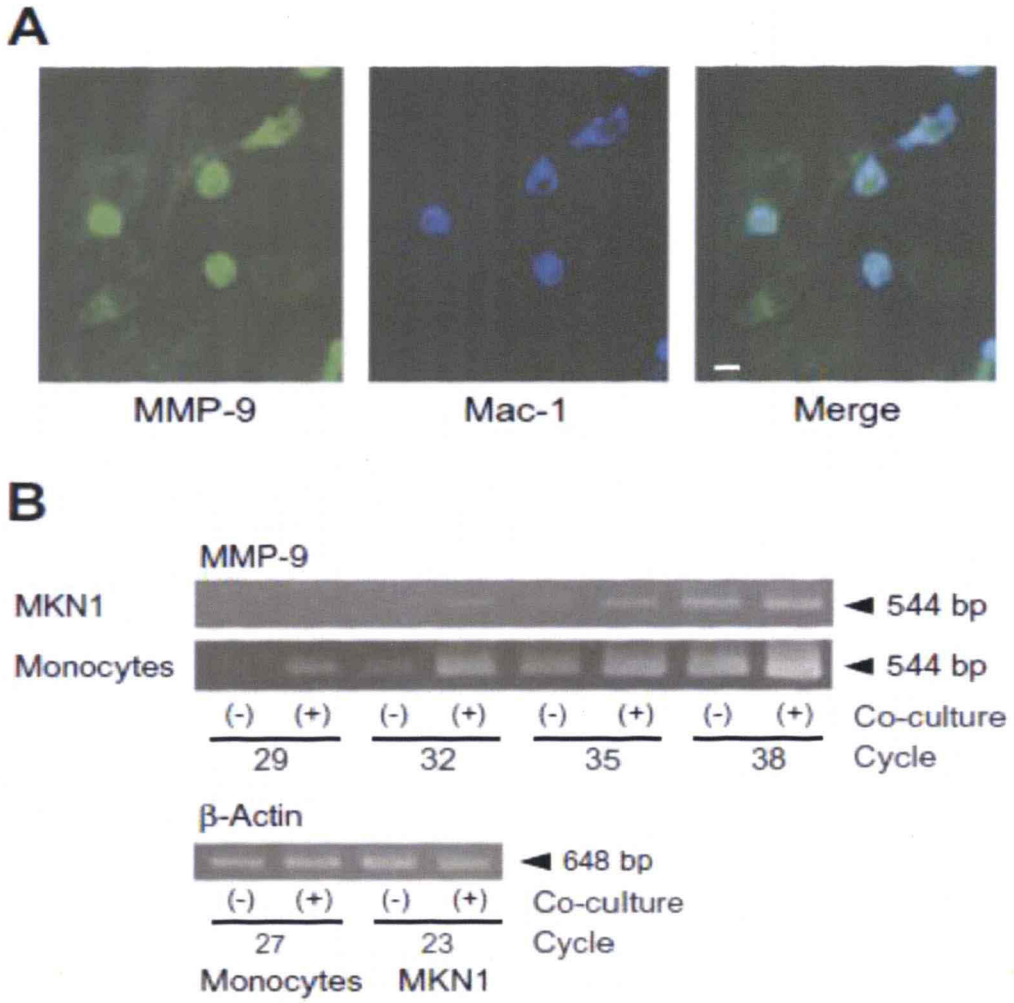

Fig. 1-2 Analyses of MMP-9-producing cells by immunofluorescence and RT-PCR. (A) Monocytes and MKN1 cells were co-cultured for 5 days, and the cells were treated with 0.2% Triton X-100/0.02% NaN_3 in PBS at 4°C for 10 min. The treated cells were then stained with anti-MMP-9 or anti-Mac-1 antibodies, followed by treatment with fluorescently labeled second antibodies. The fluorescently labeled cells were observed with a confocal laser-scanning microscope. Scale bar = 10 μm. (B) MMP-9 mRNA in monocytes and MKN1 cells after co-culture for 5 days in Boyden chambers, analyzed by RT-PCR. Amplification cycles for PCR varied from 29 to 38.

In vitro invasion of tumor cells in the presence of monocyte-derived and MMP-9-producing cells

We then assessed the effects of monocyte-derived and MMP-9-producing cells on invasion of MKN1 carcinoma cells into Matrigel basement membranes. Monocytes were first co-cultured with MKN1 or EJ-1 cells for 5 days in a Boyden chamber system (membrane pore size, 1.0 μm). Differentiated monocytes were recovered from the chamber and were mixed with fluorescently labeled MKN1 cells. The cell mixture was then placed in the upper chamber of another set of Boyden chambers (membrane pore size, 8.0 μm), the membrane between the upper and lower chamber of which had been coated with Matrigel. After the chambers were incubated at 37°C for 18 h, MKN1 cells that had migrated through Matrigel-coated membranes increased in number in the presence of monocytes that had been co-cultured with MKN1 cells (Fig. 1-3). In contrast, in the presence of monocytes that had been co-cultured with EJ-1 cells, no significant increase in the number of invading MKN1 cells was observed. The enhancing effect achieved with differentiated monocytes was correlated with MMP-9 productivity (Fig. 1-1). Furthermore, augmentation by differentiated monocytes was reversed by the addition of an MMP-9 inhibitor (Fig. 1-3), but not an MMP-2 inhibitor (data not shown). These results indicate that the invasion of MKN1 cells into the Matrigel had been promoted in the presence of differentiated monocytes in an MMP-9-dependent manner.

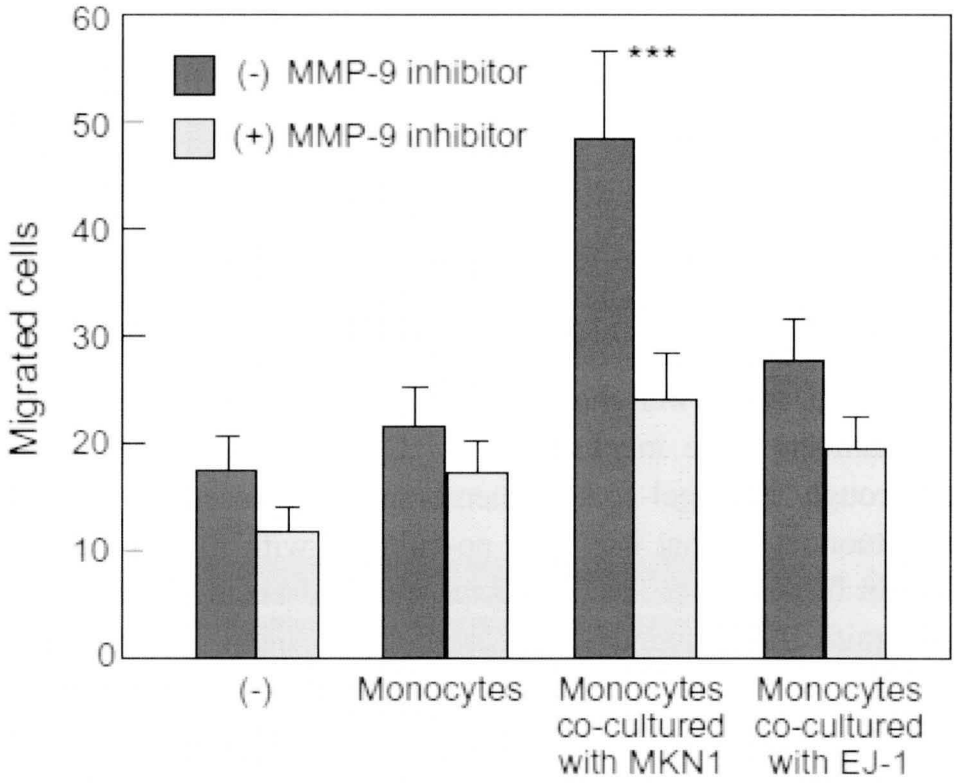

Fig. 1-3 Effects of differentiated monocytes on the invasion of MKN1 cells. The *in vitro* invasion assay was performed using a Boyden chamber system with a Matrigel-coated membrane. Monocytes (5 x 10^5 cells) were added to the lower chamber of the Boyden chamber system (pore size, 1.0 μm), and MKN1 or EJ-1 cells (2 x 10^5 cells) were added to the upper chamber. After the chambers had been incubated for 5 days, differentiated monocytes in the lower chamber were recovered. These differentiated cells (1 x 10^5 cells) were mixed with a suspension of MKN1 cells (1.25 x 10^5 cells in serum-free RPMI 1640) that had been labeled with CellTracker™ (1 μM at 37°C for 30 min), and the cell mixture was placed in the upper chamber of another set of chambers in a Boyden chamber system (pore size, 8.0 μm). The lower chamber was filled with RPMI 1640/10% FCS, and the chambers were incubated at 37°C for 18 h. MKN1 cells that had migrated through the membrane were counted under a fluorescence microscope. The data are shown as the means with SD of 6-9 fields selected at random. The assay was conducted in triplicate in the presence (*gray bar*) or absence (*black bar*) of an inhibitor of MMP-9 (1 μM). *** $p<0.005$ vs. control monocytes.

Morphological changes in monocytes induced by co-culture with tumor cells

Monocytes were co-cultured with tumor cells (MKN1, A172, SN12C, or EJ-1) in the Boyden chambers for 5 days, and then the cells cultured in this manner were observed under a phase-contrast microscope. Monocytes co-cultured with MKN1 or A172 cells were found to spread and exhibited a macrophage-like morphology, whereas after co-culture with SN12C or EJ-1 cells, most of the monocytes maintained their spherical shape and showed less flattening (Fig. 1-4A). The morphological change seen in monocytes induced by co-culture with tumor cells was correlated with MMP-9 productivity, i.e., monocytes co-cultured with MKN1 or A172 cells became polygonal and more adherent, and they produced high levels of MMP-9, whereas monocytes co-cultured with SN12C or EJ-1 cells showed no notable morphological changes and only limited MMP-9 production (Figs. 1-1 and 1-4A). We next examined expression profiles of macrophage markers on differentiated monocytes by flowcytometry. The population with high CD14 expression increased after co-culture with tumor cells (MKN1 and EJ-1), and the increase in CD14-high expresser was more prominent when monocytes were co-cultured with MKN1 cells (Fig. 1-4B). By contrast, the increase in CD68 expression on differentiated monocytes was limited.

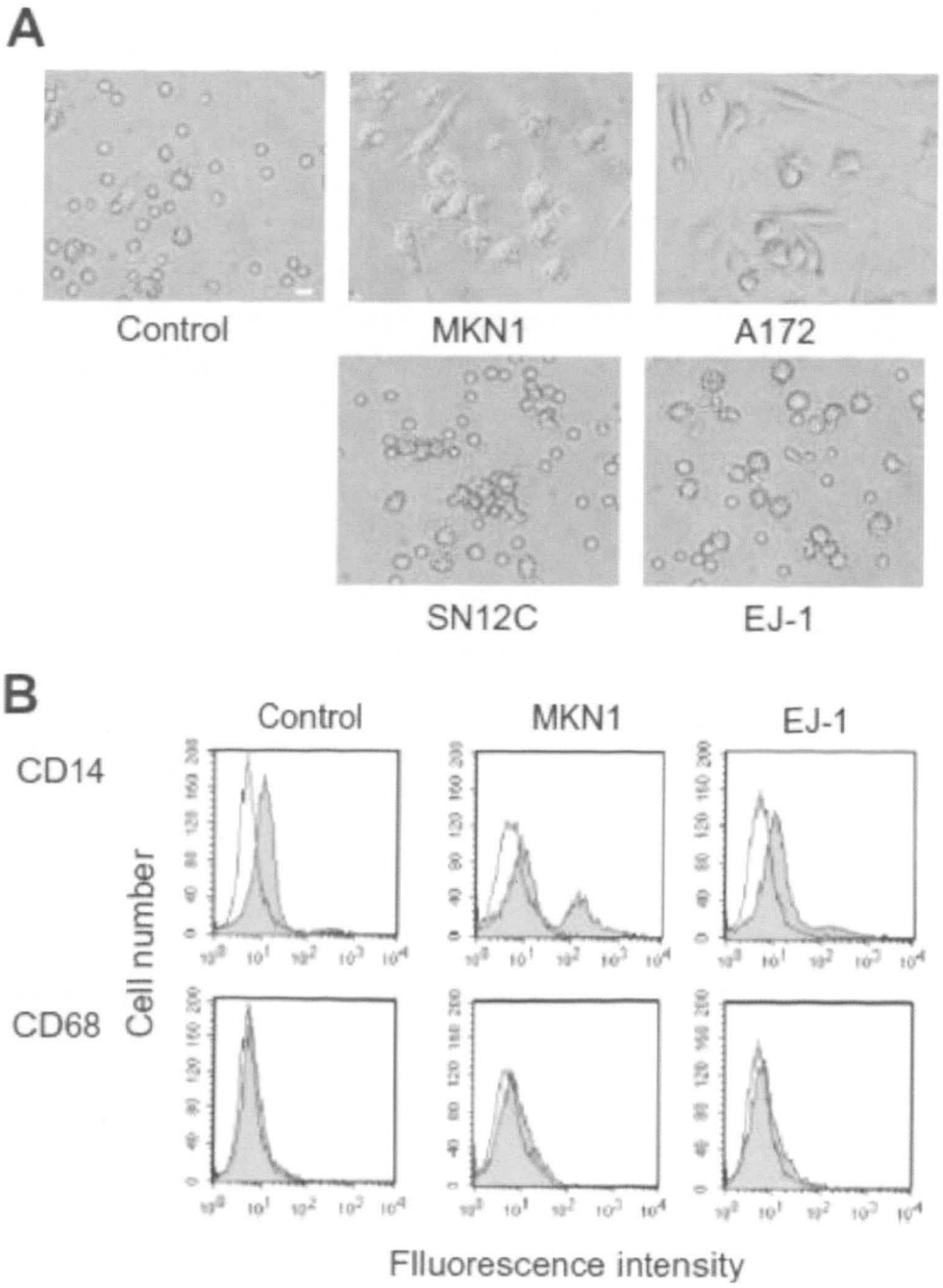

Fig. 1-4 Phenotypic changes of monocytes induced by co-cultured with tumor cells. (A) Monocytes (1-2 x 10^5 cells) were co-cultured with MKN1, A172, SN12C, or EJ-1 cells (2 x 10^5 cells) in the Boyden chamber system (pore size, 1.0 μm) as described in Fig. 1-3. After the chambers were incubated for 5 days, monocytes in the lower chamber were observed under a phase-contrast microscope. Scale bar = 10 μm. (B) Monocytes co-cultured with MKN1 or EJ-1 cells in the Boyden chamber system for 5 days were analyzed by flow cytometry after being stained with monoclonal antibody against CD14 or CD68 and with FITC-labeled secondary antibody. The control monocytes were cultured in the absence of tumor cells for 5 days. The profiles without antibody staining are indicated by lines without shadows.

Role of RGD sequence-dependent cell adhesion in the differentiation of monocytes

Because monocytes became more adhesive to culture plates during co-culture with tumor cells, we examined the possible roles of cell adhesion to ECM proteins in the process of monocyte differentiation. When monocytes were co-cultured with MKN1 cells in the presence of a GRGDSP peptide (an inhibitor of integrin-mediated cell adhesion), such monocyte spreading was no longer observed (Fig. 1-5A). However, the addition of a control GRADSP peptide showed no such inhibition of morphological change. In parallel with the inhibition of cell spreading, MMP-9 production induced by co-culture with MKN1 cells was also suppressed by the addition of a GRGDSP peptide, but not by the GRADSP peptide, either in co-culture for 5 days or in subsequent co-culture with fresh medium for 24 h (Fig. 1-5B). Similar inhibitory effects of a GRGDSP peptide on MMP-9 production were observed when monocytes were cultured with EJ-1 cells (data not shown). These results suggest that RGD-dependent cell adhesion played a critical role in bringing about the phenotypic changes induced by co-culture of these monocytes with tumor cells.

The RGD sequence is present in various ECM proteins such as fibronectin and laminin, and it plays an essential role in integrin-ECM interactions [32,44-46]. The conditioned medium of MKN1 cells contained a higher concentration of fibronectin than did the conditioned EJ-1 cell medium, as was indicated by both immunoblot and RT-PCR analyses (data not shown). In addition, a major portion of differentiation-inducing activity in the conditioned medium of MKN1 cells was recovered in a high-molecular-weight fraction after ultrafiltration with Microcon™ YM-50 (Millipore Corporation, Billerica, MA) (data not shown). We therefore assessed the effect of fibronectin depletion from the conditioned media on monocyte differentiation. The treatment of conditioned medium of MKN1 cells with gelatin-Sepharose effectively removed fibronectin, as detected by immunoblot analysis (Fig. 1-6A). When the monocytes were cultured in the fibronectin-depleted conditioned medium of MKN1 cells for 5 days, they produced a lower level of MMP-9 than did monocytes cultured with untreated conditioned medium (Fig. 1-6B). A similar reduction in MMP-9 production was observed after the culture was continued for 24 h using fresh, serum-free medium. The depletion of fibronectin from the conditioned medium of EJ-1 cells also resulted in a similar reduction in MMP-9 production (data not shown).

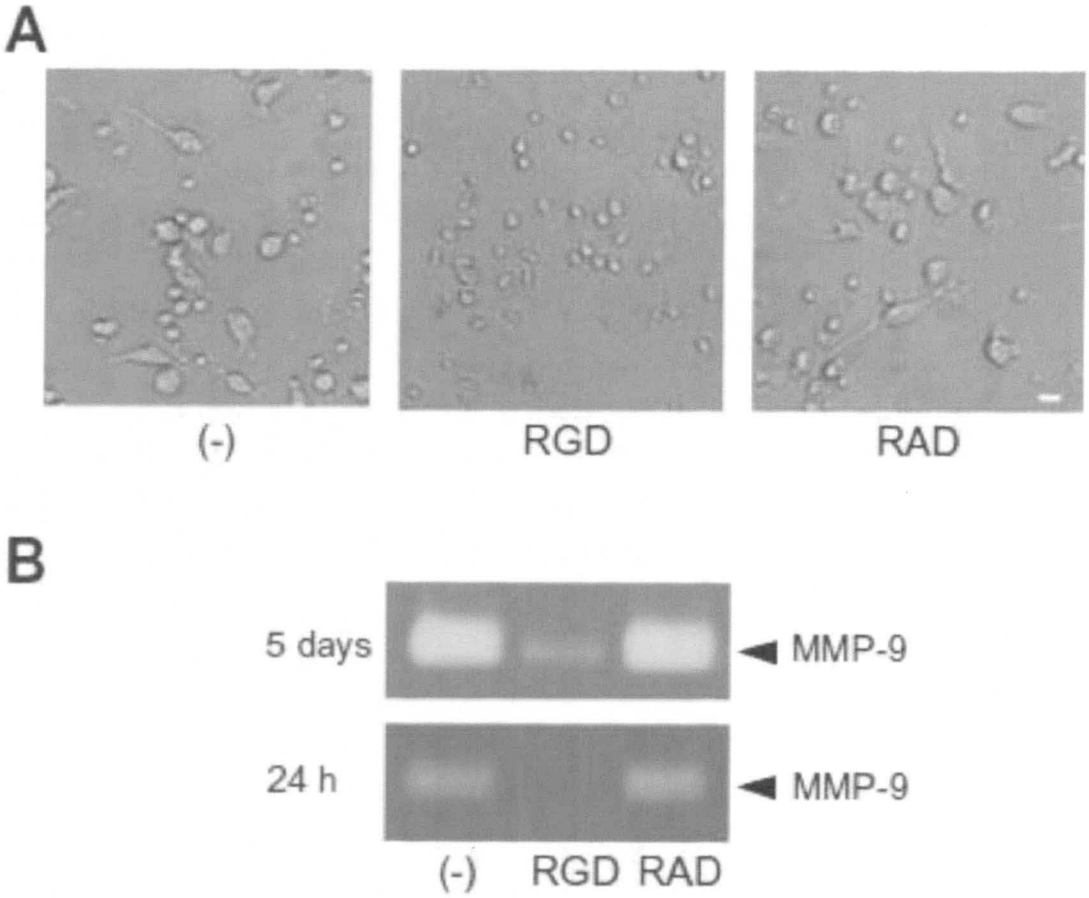

Fig. 1-5 Role of RGD-dependent cell adhesion in phenotypic changes in monocytes. (A) Monocytes (1-2 x 10^5 cells) in the lower chamber and MKN1 cells (2 x 10^5 cells) in the upper chamber of the Boyden chamber system were co-cultured in serum-free RPMI 1640 for 5 days in the presence of GRGDSP (*RGD*) or GRADSP (*RAD*) peptides (1 mM). Monocytes were observed under a phase-contrast microscope. Scale bar = 10 μm. (B) After co-culture for 5 days, described above, the culture supernatants were assayed for MMPs by gelatin zymography (*upper panel*). The co-culture was continued with fresh RPMI 1640 (serum-free) for 24 h, and the culture supernatant was again subjected to gelatin zymography (*lower panel*).

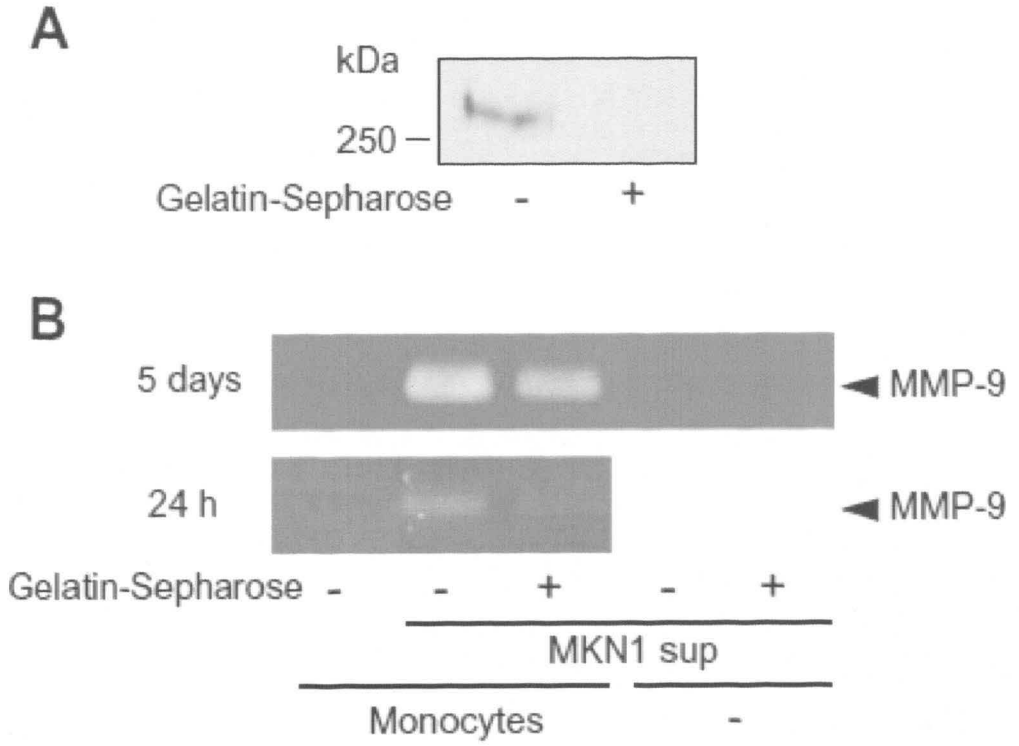

Fig. 1-6 Depletion of fibronectin from conditioned MKN1-cell medium. (A) The conditioned medium of MKN1 cells was mixed with gelatin-Sepharose, and the mixture was incubated at 4°C for 24 h. After the sedimentation of gelatin-Sepharose, the supernatant was applied to immunoblotting analysis using anti-fibronectin antibody. (B) Monocytes were cultured for 5 days in RPMI 1640 medium (serum-free) containing fibronectin-depleted or untreated conditioned MKN1-cell medium (70%). The culture supernatant was subjected to gelatin zymography (*upper panel*). The culture was continued for 24 h with fresh RPMI 1640 (serum-free), and the culture supernatant was again subjected to gelatin zymography (*lower panel*).

We next examined the direct effects of the addition of several ECM components, including fibronectin. After the lower chambers of the Boyden chamber system were coated with fibronectin, laminin-1, or Matrigel, monocytes and tumor cells (MKN1 or EJ-1 cells) were added to the lower and to the upper chambers, respectively. The co-culture was conducted in serum-free RPMI 1640 for 5 days, and monocytes cultured in a fibronectin-coated plate exhibited better spreading and a more macrophage-like morphology than did monocytes cultured in laminin-1- or Matrigel-coated, or uncoated plates (Fig. 1-7A). Coating of the plate with fibronectin also resulted in a potentiation of MMP-9 production by the monocytes after they had been co-cultured with MKN1 or EJ-1 cells (Fig. 1-7B). It should be noted that the plate-coating with laminin-1 or Matrigel abrogated the MMP-9 production induced by the co-culture with EJ-1 cells. It is suggested that monocyte differentiation was promoted by immobilized fibronectin but suppressed by laminin-1 or Matrigel.

Finally, we assayed the *in vitro* invasion of tumor cells into Matrigel in the presence of monocytes differentiated in fibronectin-coated plates. Invasion of MKN1 cells into the Matrigel was most effectively enhanced in the presence of monocytes that had been differentiated in fibronectin-coated plates by co-culture with either MKN1 or EJ-1 cells (Fig. 1-7C); this finding was consistent with the MMP-9 productivity of the differentiated monocytes.

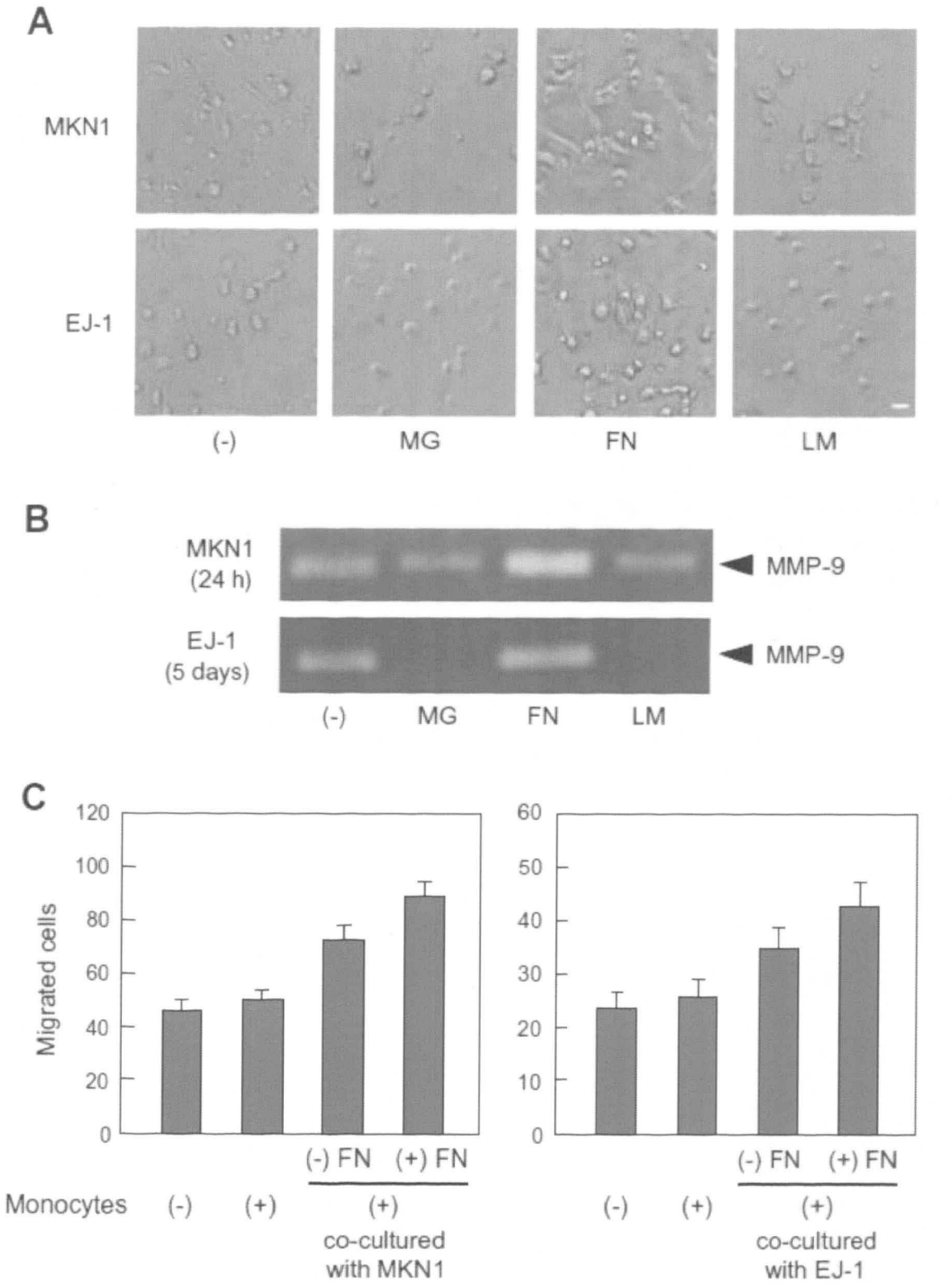

Fig. 1-7 Differentiation of monocytes by co-culture with tumor cells in ECM protein-coated plates. (A) Monocytes were cultured with MKN1 or EJ-1 cells in the Boyden chamber system. A monocyte suspension (1-2 x 10^5 cells/0.7 ml) in RPMI 1640 (serum-free) was placed in the lower chamber, which had been coated with fibronectin (*FN*), laminin-1 (*LM*),

or Matrigel (*MG*) at 4°C for 16 h. MKN1 or EJ-1 cells (2 x 10^5 cells/0.3 ml) were added to the upper chamber, and these cells were cultured for 5 days. Monocytes in the lower chamber were then observed under a phase-contrast microscope. Scale bar = 10 μm. (B) Co-culture of monocytes and tumor cells was conducted for 5 days, and then was continued for 24 h following replacement with fresh medium. The supernatants of the co-cultures were collected and assayed for MMP-9 by gelatin zymography. The results of the 5-day culture with EJ-1 cells and the 24-h culture with MKN1 cells are shown. (C) Invasion of MKN1 cells in the presence of differentiated monocytes was assayed using the Boyden chamber system. MKN1 cells (1.25 x 10^5 cells) labeled by CellTracker™ (1 μM) at 37°C for 30 min were mixed with monocytes differentiated by co-culture with MKN1 or EJ-1 cells (1 x 10^5 cells) in fibronectin-coated plates, as described in (A). The cell mixture was placed in the upper chamber equipped with a membrane that had been coated with Matrigel, and the lower chamber was filled with RPMI 1640/10% FCS. After the chambers were incubated at 37°C for 18 h, labeled MKN1 cells that had migrated through the membrane were counted under a fluorescence microscope. The assays were carried out in triplicate, and the data are presented as mean ± SD.

Discussion

In this study, we attempted to differentiate monocytes *in vitro* into TAM-like cells, and successfully induced monocytes to produce high levels of MMP-9 by co-culture with tumor cell lines and without the exogenous addition of cytokines such as M-CSF (Figs. 1-1 and 1-2). The acquisition of MMP-9 productivity by monocytes appeared to be accompanied by morphological changes, i.e., macrophage-like spreading (Fig. 1-4). These phenotypic changes induced by the co-culture depended on the type of tumor cells used. The degree of MMP-9 production and morphological changes were well correlated. The differential ability of tumor cells with regard to the induction of monocyte differentiation may reflect variation among TAMs in different patients [47,48]. MMP-9 production is considered as one of the most important characteristics of TAMs affecting both the malignant behavior of tumor cells as well as angiogenesis [7,35]. Since it is known that MMP-9 degrades type IV collagen-rich basement membranes and assists with tumor invasion and metastasis, we assayed the *in vitro* invasion of MKN1 human gastric carcinoma cells into Matrigel. The results revealed that the presence of differentiated monocytes promoted the invasion of MKN1 cells in an MMP-9-dependent manner (Fig. 1-3). Moreover, the promotion of cell invasion by differentiated monocytes was correlated with their MMP-9 productivity. Thus, the results clearly indicate that monocyte-derived and TAM-like cells promote tumor cell invasion.

MMP-9 secretion from monocytes was detected after 3-day co-culture of the monocytes with tumor cells, and levels of secretion gradually increased for up to 7 days of culture (Fig. 1-1). Monocytes freshly isolated from blood were readily differentiated into cells that produced high levels of MMP-9 within a rather short period of culture, and this differentiation required no prior preparation of monocyte-derived macrophages [23,27]. The co-culture of monocytes and tumor cells in the Boyden chamber culture system indicated an essential role of soluble factors secreted from tumor cells, as well as the importance of cell-to-cell contact, in monocyte differentiation. A recent study by Solinas and co-workers [24] demonstrated that conditioned media of tumor cells induced the differentiation of monocytes. They also found that the gene expression profiles of these differentiated monocytes (designated as "tumor-conditioned (TC) macrophages") were similar to those of TAMs. The differentiated monocytes seen in this study may thus share common phenotypes

with such TC-macrophages. Their study also indicated that the differentiation of TC-macrophages involved M-CSF as an essential factor. In the present study, we demonstrated that RGD-dependent cell adhesion plays a critical role in monocyte differentiation. Fibronectin produced by tumor cells was a strong candidate involved in cell adhesion. The most typical RGD-dependent cell adhesion was found to be mediated by the interaction between fibronectin and α5β1 integrin [32]. Terui and co-workers [37] reported that α5β1 integrin was upregulated during monocyte differentiation, and this cell adhesion molecule may function as a receptor for fibronectin, leading to the promotion of monocyte differentiation. One possibility is that α5β1 integrin-fibronectin interaction mediates cell-to-cell contact between monocytes and tumor cells, causing an effective differentiation of monocytes (Fig. 1-1C). However, it was of note that neither laminin-1 nor Matrigel exerted a positive effect on this differentiation. On the contrary, Matrigel and laminin-1 interestingly decreased the MMP-9 expression levels. These results suggest that specific interactions between ECM proteins and their cellular receptors are required for the differentiation process.

Macrophages are divided into two populations, which are respectively referred to as M1 and M2 according to phenotype, including the cytokine production profile [20]. The M1 type is a classic immunoactive macrophage and produces IL-12 and TNF-α, whereas the M2 type is involved in tissue remodeling and produces IL-10, but not IL-12. TAMs are considered to be polarized towards M2-type cells with immunosuppressive functions such as poor antigen-presenting capacity and the suppression of T-cell activation, in addition to a production of growth and angiogenic factors and MMPs. It is therefore likely that the monocytes in the present study underwent differentiation directed toward M2-like cells during the co-culture with tumor cells. Although the characteristics of TAMs are generally thought to be heterogeneous, the phenotypes of the TAM-like cells differentiated from blood monocytes in this study will still need to be analyzed in detail in future studies. The TAM-like cells induced in the present *in vitro* system provide a useful model to investigate the process of differentiation in TAMs, and will help efforts to characterize the functions of TAM in modulating the malignant behavior of tumor cells.

The person can become both a good guy and a poor guy, by environment and education.

Chapter 2

Potentiation of tumor cell invasion by co-culture with monocytes accompanying enhanced production of matrix metalloproteinase and fibronectin

Introduction

The malignant behaviors of tumor cells are greatly influenced by the peritumoral microenvironment. The mutual interactions between tumor cells and cellular and non-cellular components (extracellular matrix: ECM) of the host microenvironment affect tumor development and progression by controlling proliferation, migration, invasion, metastasis, angiogenesis, and apoptosis [1-3]. Macrophages are a major population of host immune cells, and a subset called tumor-associated macrophages (TAMs) infiltrate into tumor tissues. Although macrophages have been thought to exhibit cytotoxicity against tumor cells, recent studies have demonstrated that TAMs enhance tumor progression [5-7,49]. Indeed, TAMs have been reported to secrete various cytokines, growth factors, and enzymes that promote tumor metastasis and angiogenesis, including TNF-α [12,13] epidermal growth factor (EGF) [10], vascular endothelial growth factor (VEGF) [8], interleukin (IL)-8 [9], platelet-derived growth factor (PDGF) [11], and matrix metalloproteinases (MMPs) [13,14]. These paradoxical activities of TAMs on tumor progression might be determined by the directions of TAM differentiation [21]. To characterize the biological nature of TAMs, we previously attempted to induce differentiation of human peripheral blood monocytes *in vitro* by co-culture with tumor cells, and succeeded in differentiating monocytes into TAM-like cells producing a high level of MMP-9 (Chapter 1). We also reported that ECM proteins such as fibronectin played an important role in the monocyte differentiation and that the differentiated monocytes promoted tumor cell invasion. During co-culture of these cells, we observed that tumor cells changed their morphology and production of fibronectin. The interaction of tumor cells with ECM proteins has been thought to be important for various aspects of tumor invasion and metastasis [50-52]. In fact, a number of studies have suggested that tumor invasion is influenced by ECM proteins such as fibronectin [53,54], laminin [55], and tenascin [56]. On the other hand, integrins, a major class of cell adhesion molecules, serve as cellular receptors for ECM proteins, and their interactions with ECM proteins play key roles in tumor cell behaviors. The integrin-mediated cell adhesion to ECM transduces signals into tumor cells to potentiate production of MMPs [57-59] and to induce tyrosine phosphorylation of focal adhesion kinase (FAK) [60].

In the present study, we focused on phenotypic changes of tumor cells after the co-culture with monocytes. We first describe an increase in the invasive potential of some tumor cells after the co-culture with monocytes in parallel with an enhanced secretion of MMP-9. Secondly, we show that the co-culture induced tumor cells to change their morphology. We then analyzed the relationship between these phenotypic changes and the ECM-integrin system, especially fibronectin and α5β1 integrin. Fibronectin is an ECM protein with wide tissue distribution and plays crucial roles in various cellular functions, including proliferation, migration, and differentiation, by interacting with integrins via an RGD (Arg-Gly-Asp) triplet sequence in its cell-binding domain. The α5β1 integrin is a prototype of an RGD-recognizing cellular receptor for fibronectin [53,61,62]. We examined the secretion of fibronectin into the culture medium and the expression of α5 integrin on the cell surface. Finally, we assessed the roles of cytokines produced by monocytes in the potentiation of tumor invasion, and present evidence that tumor necrosis factor (TNF)-α is at least partly involved in the potentiation of tumor cell invasion after co-culture with monocytes.

Materials and methods

Cells

MKN1 (a human gastric carcinoma cell line) was provided by RIKEN cell bank (Tsukuba, Japan). EJ-1 (a human bladder carcinoma cell line), HT1080 (a human fibrosarcoma cell line), and A172 (a human glioma cell line) were provided by the Human Science Research Resources Bank (Osaka, Japan). SN12C (a human renal carcinoma cell line) was donated by Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center). These cells were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal calf serum (FCS) at 37°C under a 5% CO_2 atmosphere.

Monocytes were isolated from human peripheral blood essentially as described previously [38]. Briefly, heparinized human blood was mixed with an equal volume of 3% dextran 200,000/saline to sediment most of the erythrocytes. After the samples were left to stand for 30 min, the supernatant was centrifuged at 1200 rpm for 10 min. Pelleted cells were then subjected to Ficoll-Paque density gradient centrifugation at 1200 rpm for 30 min, and mononuclear cells (PBMCs) were recovered. The PBMCs thus obtained were allowed to adhere to the culture dish in RPMI 1640/10% FCS at 37°C for 1 h, and adherent cells were collected by a cell scraper. The purity of monocytes was estimated to be 60-80% by flow cytometry (forward scattering/sidescattering plots).

Reagents and antibodies

Heparin was purchased from Novo Nordisk (Copenhagen, Denmark). Dextran 200,000 and Ficoll-Paque™ PLUS were obtained from Wako Chemicals Industries (Osaka, Japan) and GE Healthcare (Piscataway, NJ, USA), respectively. Matrigel™ was purchased from BD Biosciences (San Diego, CA, USA). Recombinant human TNF-α was purchased from PeproTech (Rocky Hill, NJ, USA). An MMP-9 inhibitor (anthranilic acid derivative), H-Gly-Arg-Gly-Asp-Ser-Pro-OH (GRGDSP) peptide, and H-Gly-Arg-Ala-Asp-Ser-Pro-OH (GRADSP) peptide were purchased from Calbiochem (San Diego, CA, USA). Protein G-Sepharose 4 FF was purchased from GE Healthcare.

Antibodies directed to fibronectin and the α5 integrin subunit were purchased from TaKaRa (Osaka, Japan) and Santa Cruz Biotechnology (Santa

Cruz, CA, USA), respectively. Anti-focal adhesion kinase (FAK) antibody and anti-FAK (pY397) antibody were purchased from BD Biosciences. Anti-vimentin and anti-β-actin antibodies were obtained from Sigma (St. Louis, MO, USA). Anti-TNF-α and anti-MMP-9 antibodies were purchased from R&D Systems (Minneapolis, MN, USA). FITC-labeled anti-mouse IgG antibody and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Kirkegaard & Perry Laboratories (KPL; Gaithersburg, MD, USA).

Co-culture of tumor cells with monocytes

Co-culture of tumor cells and monocytes was conducted in a Boyden chamber system as described previously (Chapter 1). Briefly, tumor cells (2 x 10^5 cells) and monocytes (1-2 x 10^5 cells) were added to the lower chamber (Falcon 3504; 24-well culture plate) and the upper chamber (Falcon 3104; cell culture insert with a PET membrane (pore size, 1.0 μm)), respectively, and the chambers were incubated at 37°C for 5 days.

In vitro invasion assay

The *in vitro* invasion assay was performed using the Boyden chamber system (Falcon 3504, 24-well culture plate; Falcon 3097, cell culture insert with PET membrane (pore size: 8.0 μm)), as described previously [42]. Tumor cells (1.25 x 10^5 cells) were suspended in RPMI 1640 (serum-free) and placed in the upper chamber. The membrane between the upper and lower chambers had previously been coated with Matrigel (0.035 ml) at 4°C for 16 h. After removal of Matrigel solution, the membrane was incubated at 37°C for 30 min to form a gel layer. The lower chamber was filled with RPMI 1640/10% FCS, and the chambers were incubated at 37°C for 18 h. Tumor cells that had migrated through Matrigel-coated membranes were counted. In some experiments, the cell invasion was assayed in the presence of an MMP-9 inhibitor (anthranilic acid derivative, 1 μM) or a GRGDSP peptide (1 mM).

Gelatin zymography

The MMP activity was detected by gelatin zymography essentially as described previously [39]. The culture supernatant was electrophoresed in a polyacrylamide gel (6.5%) containing SDS (0.1%) and gelatin (1.5 mg/ml)

under nonreducing conditions. The gel was washed three times with washing buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, pH 7.5) containing 2.5% Triton X-100 for 30 min, and incubated in washing buffer lacking Triton X-100 at 37°C for 16 h. The gel was then stained with Coomassie brilliant blue (CBB) and destained with 7.5% acetic acid/5% methanol.

Immunoblotting

The culture supernatants were subjected to SDS-polyacrylamide gel (6.5%) electrophoresis, and separated proteins were electrically blotted onto a nitrocellulose membrane (Hybond-ECL; GE Healthcare) using a semi-dry blotting system (BE-330; Bio-Craft, Tokyo, Japan). After the blotted membrane was blocked with 4% BlockAce™ (a milk protein-based blocking reagent; Dainippon Pharmaceutical, Osaka, Japan) for 30 min, the membrane was treated with anti-fibronectin (1:1000 dilution) or anti-MMP-9 antibody (1:1000 dilution) for 1 h and then with HRP-conjugated secondary antibodies (anti-mouse or anti-goat IgG antibody, 1:1000 dilution) for 40 min. The membrane was washed with PBS containing 0.05% Tween 20 (PBS-T) three times after each reaction step. Proteins were detected with an enhanced chemiluminescence (ECL) detection system (GE Healthcare).

Intracellular proteins were also analyzed by immunoblotting essentially under the same conditions as those described above. The sample was prepared as follows: cells were incubated in the RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS) at 4°C for 15 min, and the lysate was centrifuged at 12,000 rpm for 15 min. The supernatant was mixed with an equal volume of sample buffer for SDS-polyacrylamide gel electrophoresis.

Flow cytometry

The expression of the α5 integrin subunits was measured with a flow cytometer (FACS Calibur; BD Biosciences) using an antibody against α5 integrin subunit and FITC-labeled anti-mouse IgG antibody as described previously [40].

Depletion of TNF-α from conditioned medium by anti-TNF-α antibody

A conditioned medium (1.0 ml) of monocyte culture in RPMI 1640 (serum-free) for 3 days was mixed with anti-TNF-α antibody conjugated with protein G-Sepharose (0.05 ml, 50% suspension) and incubated at 4°C for 16 h. The supernatant was recovered after low-speed centrifugation and sterilized with a membrane filter.

Results

Potentiation of tumor cell invasion and MMP secretion by co-culture with monocytes

MKN1 cells were co-cultured with monocytes in a Boyden chamber system for 5 days and subjected to *in vitro* invasion assay. The migration of MKN1 cells through Matrigel-coated membranes was increased by 2.0-fold after the cells were co-cultured with monocytes (Fig. 2-1A). The enhanced invasion of MKN1 cells was decreased almost to the control level in the presence of an MMP-9 inhibitor. When the serum-free conditioned media of MKN1 cells were analyzed by gelatin zymography and immunoblotting, the secretion of MMP-9 was greatly elevated after the co-culture with monocytes (Fig. 2-1B). However, the secretion of MMP-2 from either co-cultured cells or control cells was at an undetectable level. We next examined whether similar enhancements of cell invasion and MMP-9 production would be observed with cell lines other than MKN1. The invasion of HT1080 cells was also potentiated by the co-culture with monocytes, but none of the other cell lines (EJ-1, A172, or SN12C) showed a significant increase in invasiveness (Fig. 2-2A). The secretion of MMP-9 from HT1080 cells into culture medium was similarly increased, whereas that from the other cell lines remained substantially unchanged (Fig. 2-2B). These results suggest a correlation between enhancement of invasive potential and MMP-9 secretion.

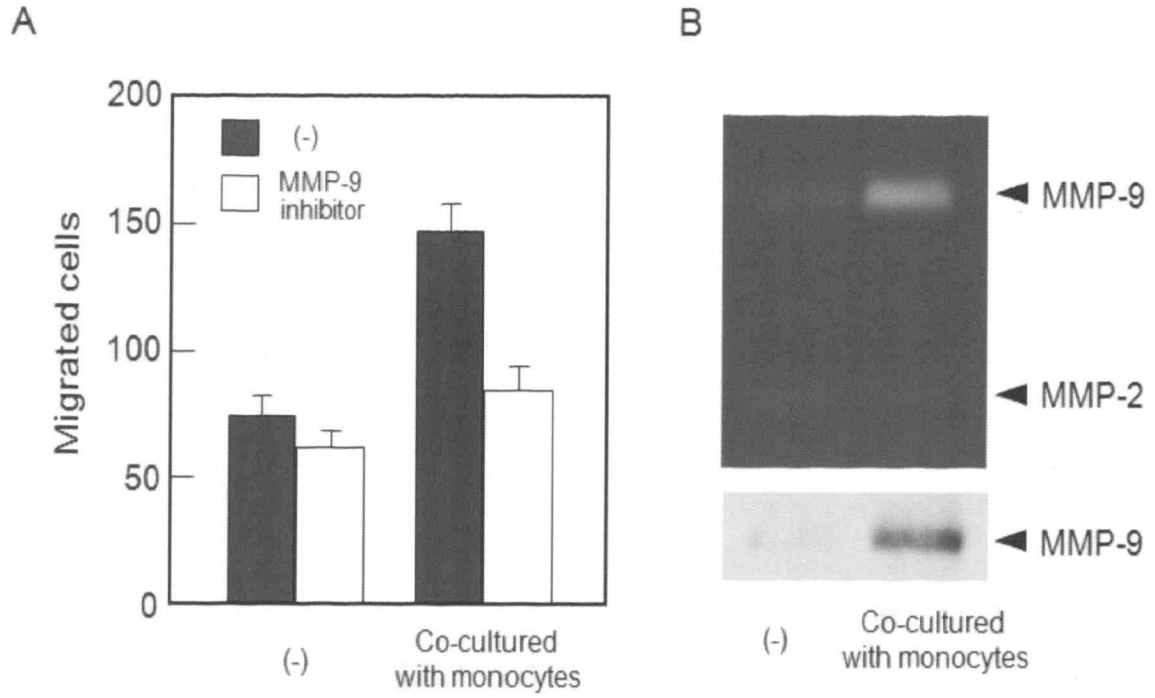

Fig. 2-1 Enhancement of MKN1 cell invasion and secretion of MMP after co-culture with monocytes. (A) The *in vitro* invasion assay was performed using a Boyden chamber system with a Matrigel-coated membrane. MKN1 cells (1.25×10^5 cells) that had been co-cultured with monocytes were suspended in serum-free RPMI 1640 with or without an MMP-9 inhibitor (1 μM), and the cell suspension (0.2 ml) was placed in the upper chamber. The lower chamber was filled with RPMI 1640/10% FCS, and the chambers were incubated at 37°C for 18 h. Cells migrated through the membrane were counted under microscopic observation. The data are shown as the means with SD of 6–9 fields selected at random. (B) MKN1 cells (2×10^5 cells) that had been co-cultured with monocytes were cultured for 48 h in serum-free RPMI 1640, and the culture supernatants were assayed for MMPs by gelatin zymography (upper panel) or by immunoblotting (lower panel).

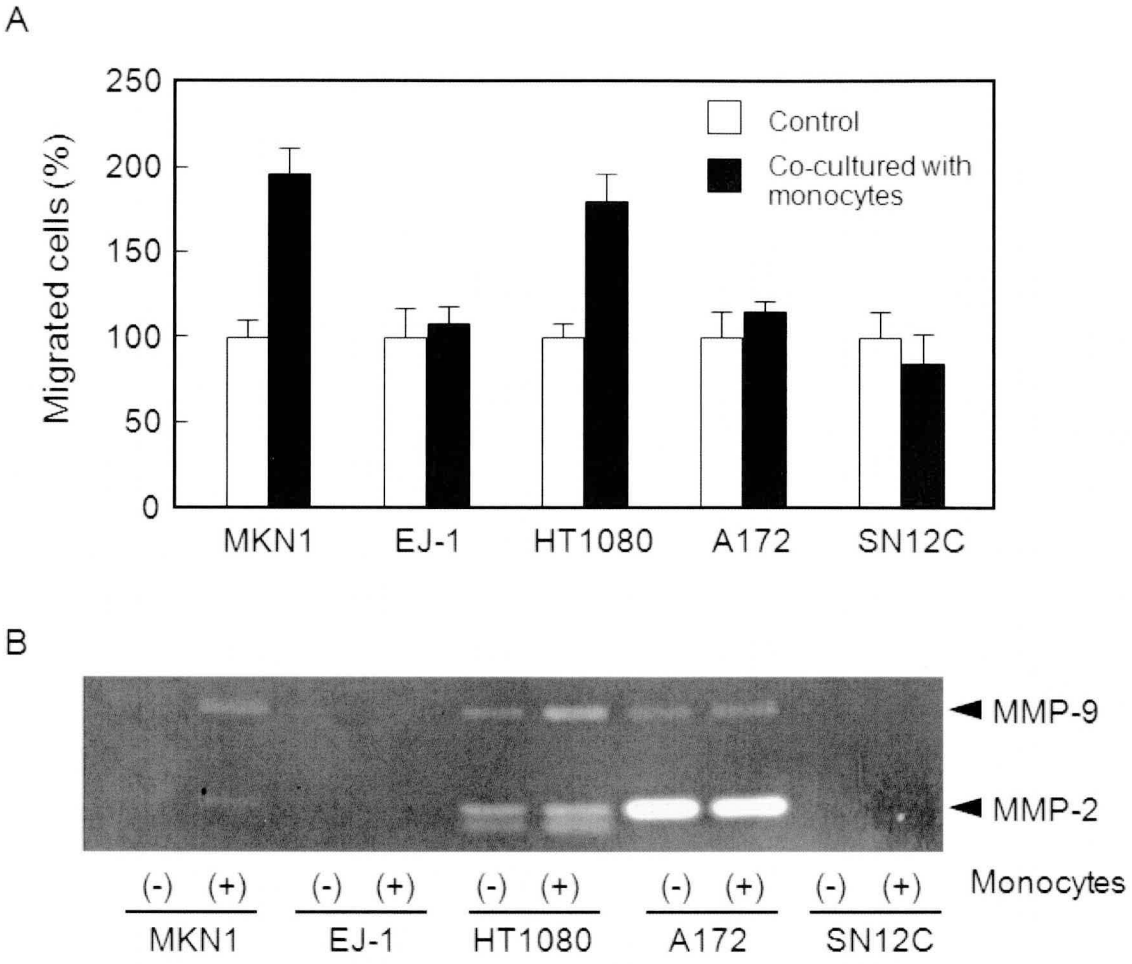

Fig. 2-2 Cell invasion and MMP productivity of various tumor cells after co-culture with monocytes. (A) The *in vitro* invasion assay was performed using a Boyden chamber system with a Matrigel-coated membrane essentially as described in Fig. 2-1. The cell migration was expressed in relation to that of the control cells without co-culture taken as 100%. The data are shown as the means with SD of 6–9 fields selected at random. (B) Tumor cells that had been co-cultured with monocytes were cultured for 48 h in RPMI 1640 (serum-free), and the culture supernatants were subjected to gelatin zymography.

Morphological change and fibronectin production after co-culture with monocytes

MKN1 cells co-cultured with monocytes for 5 days in RPMI 1640 (serum-free) exhibited a rather spread-out and flattened morphology as compared with those of the control culture (Fig. 2-3A). Because the tumor cells appeared to become more adherent to the plate, we thought that ECM proteins may have been involved and examined the secretion of fibronectin into the culture medium by immunoblotting. The concentration of fibronectin in the conditioned medium of co-cultured MKN1 cells and monocytes was found to be much higher than that from single culture of either MKN1 cells or monocytes (Fig. 2-3B). We then performed immunoblotting to compare the production of fibronectin in MKN1 cells cultured in the presence or absence of monocytes. As shown in Fig. 2-3C, cellular fibronectin of MKN1 cells was also markedly increased after co-culture with monocytes (Fig. 2-3C).

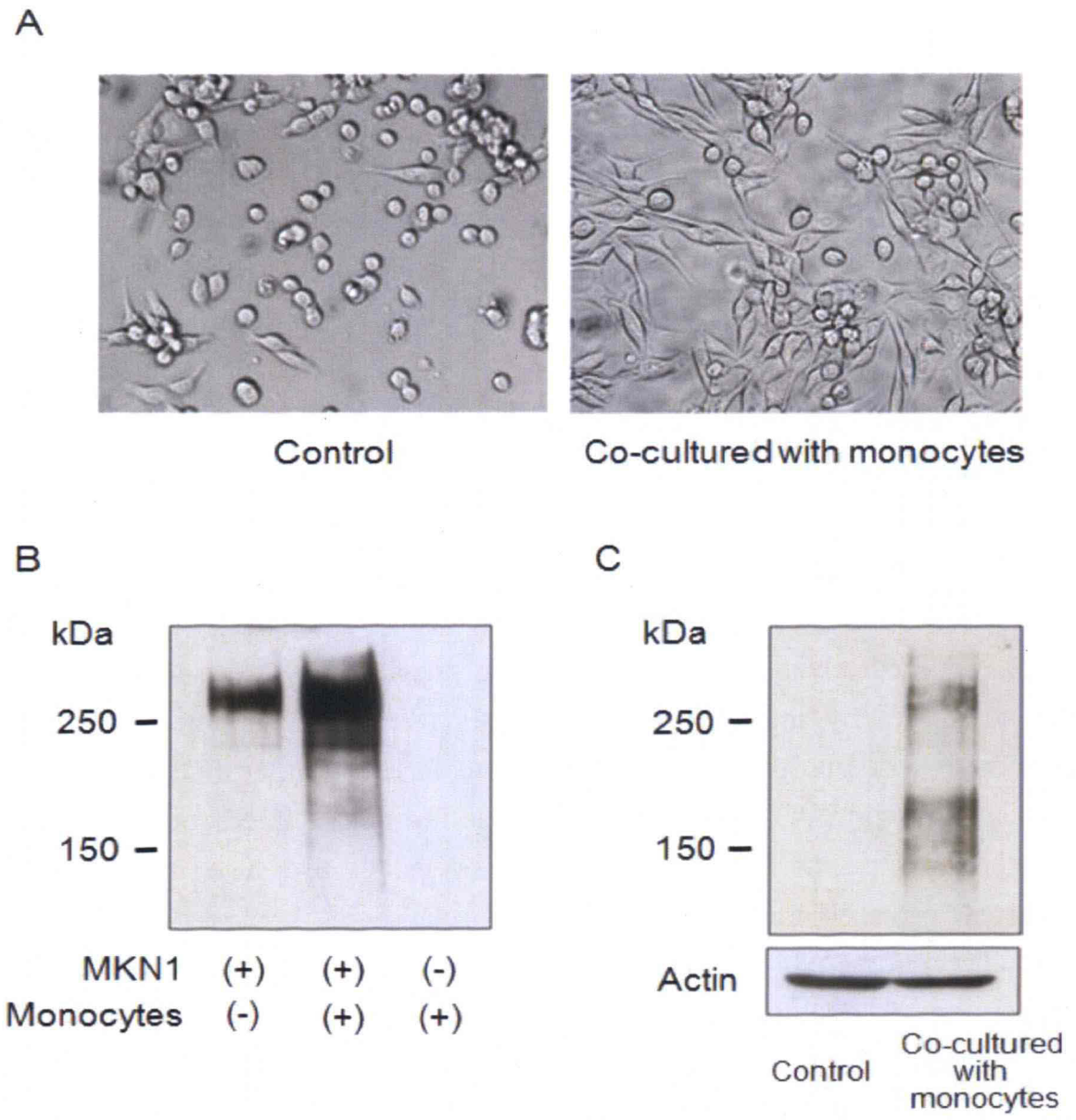

Fig. 2-3 Morphological change and fibronectin production induced by the co-culture with monocytes. (A) MKN1 cells (2 x 10^5 cells) were co-cultured with monocytes (1-2 x 10^5 cells) in RPMI 1640 (serum-free) in the Boyden chamber system for 5 days, and observed under a phase-contrast microscope. (B) MKN1 cells were co-cultured with monocytes as described above, and the conditioned medium was assayed for fibronectin by immunoblotting. The conditioned media of single culture of MKN1 cells or monocytes were also analyzed. (C) Levels of fibronectin (upper panel) and actin (used as a control; lower panel) in MKN1 cells that had been co-cultured with monocytes were analyzed by immunoblotting.

Role of the fibronectin-integrin interaction in the increased tumor cell invasion

To evaluate the role of the fibronectin-integrin interaction in the tumor cell invasion potentiated by the co-culture with monocytes, we examined the effects of an integrin-inhibiting RGD peptide. The enhanced invasion of MKN1 cells that had been co-cultured with monocytes was inhibited in the presence of a GRGDSP peptide (1 mM) to the control level (Fig. 2-4A). However, the addition of a control GRADSP peptide resulted in no such inhibition. These results suggest that the fibronectin-integrin interaction plays a role in the tumor cell invasion potentiated by monocytes. We next measured the change in the expression of α5 integrin, which constitutes a cellular receptor for fibronectin. The flow cytometric analysis showed that α5 integrin expression on the MKN1 cell surface was increased after co-culture with monocytes (Fig. 2-4B). We then examined whether the focal adhesion kinase (FAK) was activated during the co-culture by immunoblotting analysis using phospho-specific anti-FAK antibody. The phosphorylation of FAK was increased after the co-culture (Fig. 2-4C), supporting the idea that the fibronectin-integrin interaction is important for the increase in invasiveness of tumor cells. Because it was recently reported that the FAK phosphorylation is accompanied by epithelial-mesenchymal transition in tumor cells [63], we also examined the expression of vimentin by immunoblotting. As shown in Fig. 2-4D, vimentin was upregulated after co-culture with monocytes. Considering that vimentin and fibronectin are mesenchymal cell markers [64,65], the MKN1 cells might have undergone mesenchymal-directed differentiation.

Among the five cell lines used in this study, MKN1 and HT1080 cells were stimulated to secrete fibronectin into culture media (Fig. 2-5A) and to express α5 integrin on their surfaces (Fig. 2-5B) by co-culture with monocytes, while EJ-1, A172, and SN12C cells were not. These patterns of fibronectin secretion and α5 integrin expression were well correlated with those of enhanced invasive potential after the co-culture with monocytes (Fig. 2-2A).

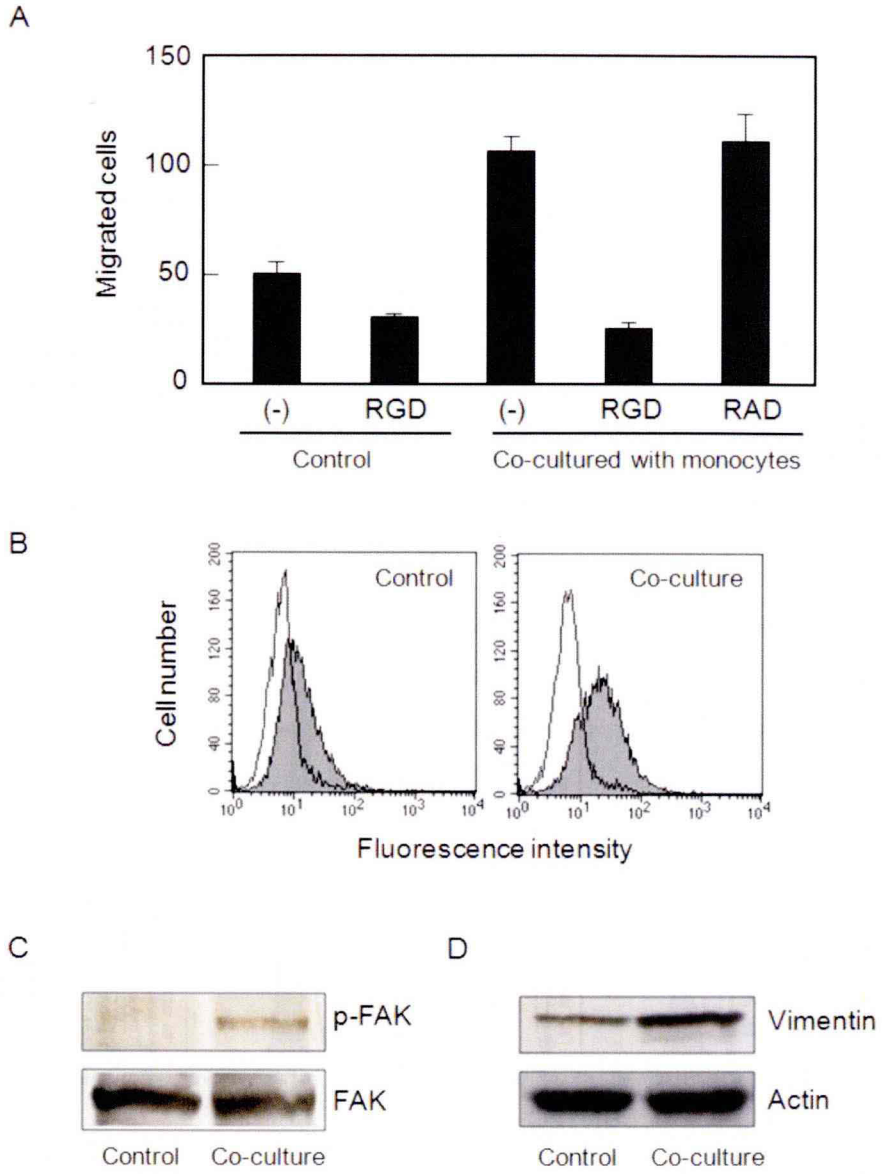

Fig. 2-4 Roles of the fibronectin-integrin interaction in tumor cell invasion. (A) MKN1 cells that had been co-cultured with monocytes were subjected to *in vitro* invasion assay in the presence or absence of integrin-inhibiting GRGDSP peptide (*RGD*, 1 mM). A GRADSP peptide (*RAD*, 1 mM) was also used as a control. The experimental conditions were as described in Fig. 2-1. (B) MKN1 cells that had been co-cultured with monocytes for 5 days were analyzed by flow cytometry after being stained with anti-α5 integrin antibody and with FITC-labeled secondary antibody. The profiles without primary antibody staining are indicated by lines without shadows. (C) Focal adhesion kinase (FAK) activation was assessed by immunoblotting using anti-phospho-specific FAK antibody (upper panel) and anti-total FAK antibody (lower panel). (D) The change in the expression of vimentin (upper panel) in MKN1 cell after the co-culture with monocytes was analyzed by immunoblotting. The expression of actin was also analyzed as a control (lower panel).

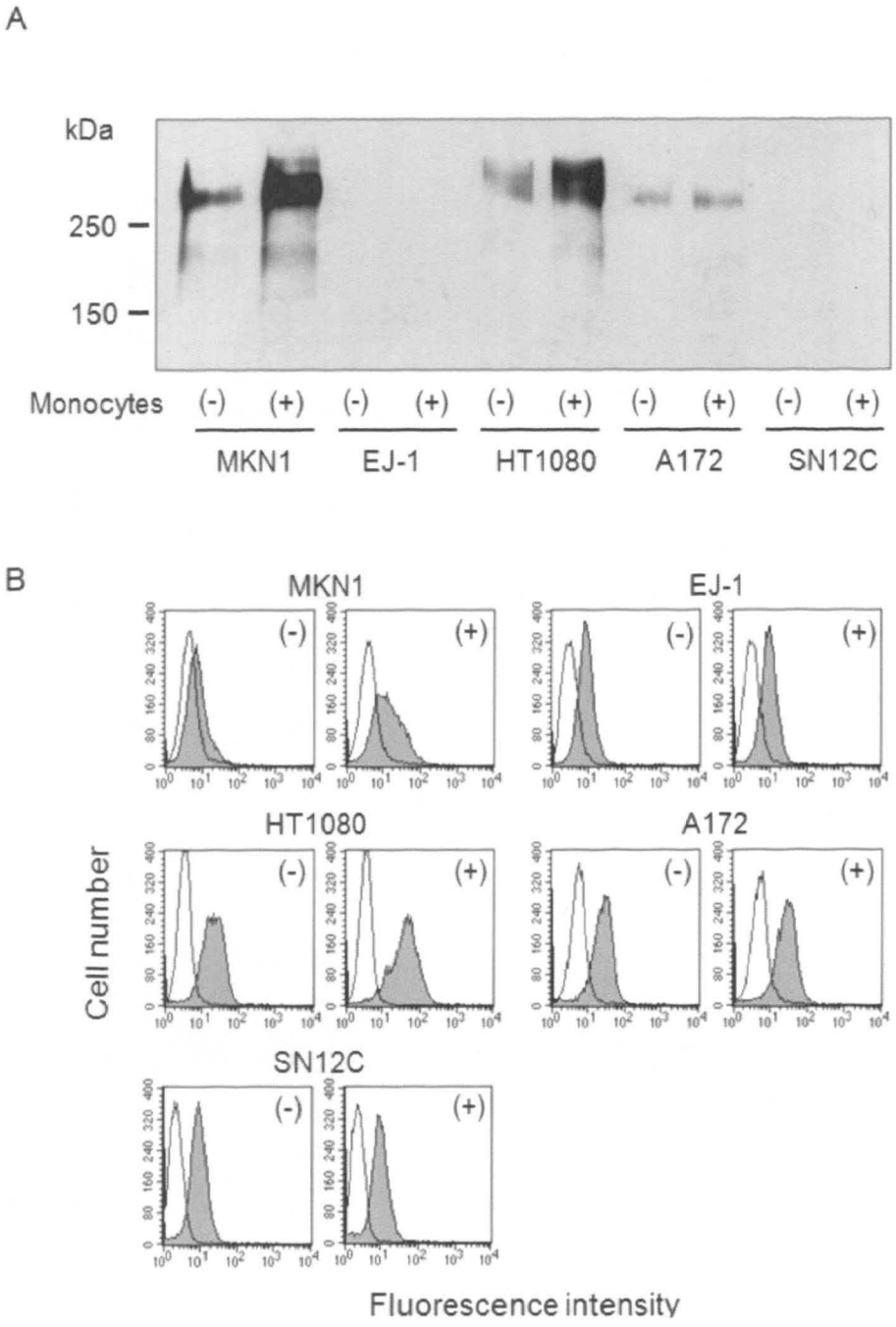

Fig. 2-5 Changes in the fibronectin secretion and α5 integrin expression after the co-culture with monocytes. (A) Five tumor cell lines were co-cultured with monocytes for 5 days, and the conditioned media were assayed for fibronectin by immunoblotting. (B) The expression of α5 integrin in tumor cells that had been co-cultured with or without monocytes was analyzed by flow cytometry. The profiles without primary antibody staining are indicated by lines without shadows.

Role of TNF-α in the changes in invasive phenotypes of tumor cells

Recent studies have indicated that inflammatory cytokines such as TNF-α accelerate the malignant behaviors of tumor cells [12,13,30,31,66]. We therefore assessed the roles of TNF-α in the enhanced tumor cell invasion. MKN1 cell invasion into Matrigel was promoted by the conditioned medium of monocytes, but the enhanced invasiveness of these cells was no longer observed after TNF-α was depleted from the conditioned medium with anti-TNF-α conjugated with protein G-Sepharose (Fig. 2-6A). In parallel with the loss of invasion-promoting activity, the secretion of MMP-9 and fibronectin was also suppressed by depleting TNF-α from the conditioned medium of monocytes (data not shown). Finally, we examined the effects of TNF-α treatment on the invasive potential of MKN1 cells. After being cultured in the presence of TNF-α (10 ng/ml) for 5 days, MKN1 cells exhibited a more invasive phenotype (Fig. 2-6B), as well as an increase in the secretion of MMP-9 (Fig. 2-6C) and fibronectin (Fig. 2-6D).

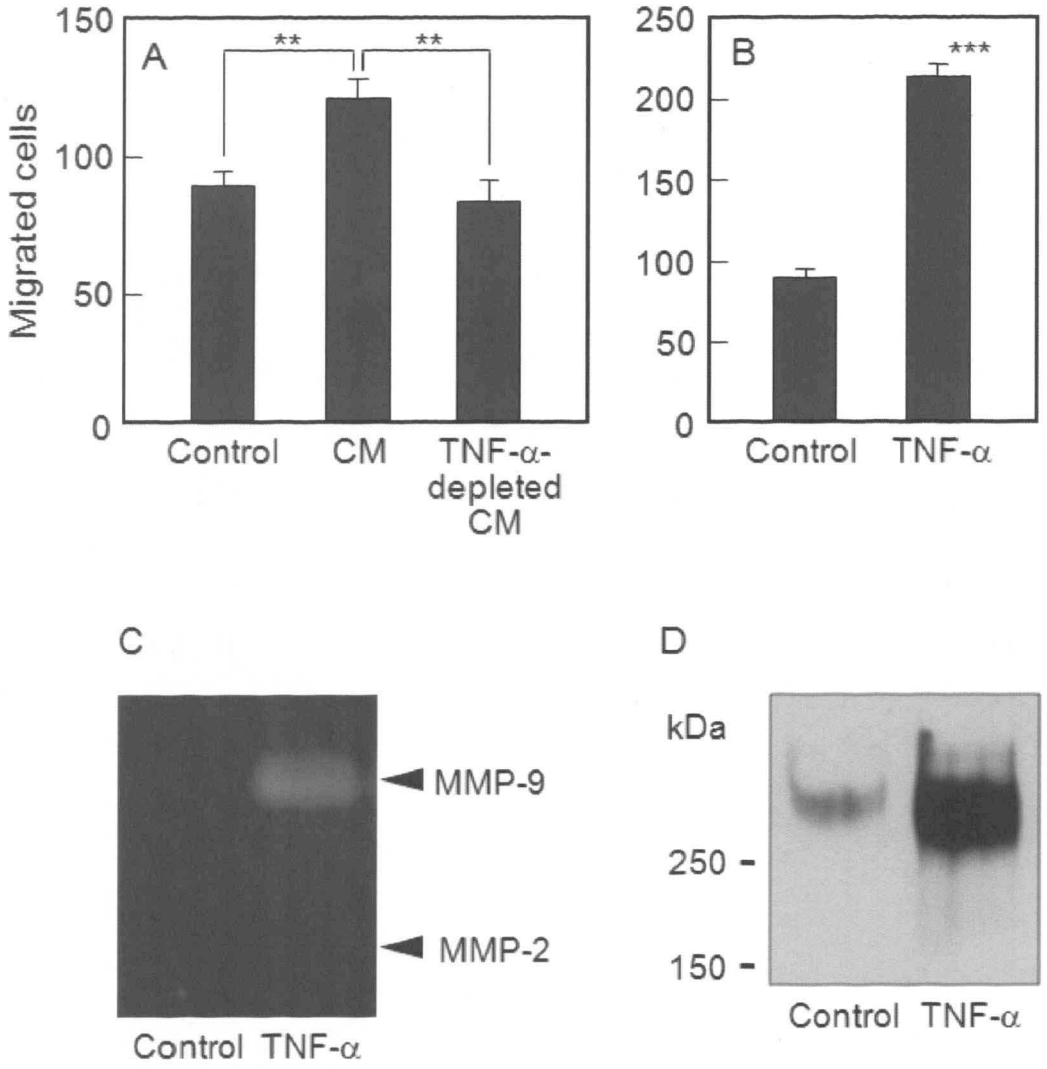

Fig. 2-6 Role of TNF-α in MKN1 cell invasion and secretion of MMP-9 and fibronectin. (A) TNF-α in the conditioned medium of monocyte culture was depleted by the treatment with anti-TNF-α antibody-conjugated protein G-Sepharose at 4°C for 16 h. MKN1 cells were cultured in control medium, conditioned medium (70%), or TNF-α-depleted conditioned medium (70%) for 5 days, and subjected to *in vitro* invasion assay. The data are shown as the means with SD of 6–9 fields selected at random. $^{**}p < 0.01$. (B) MKN1 cells that had been treated with TNF-α (10 ng/ml) for 5 days were subjected to *in vitro* invasion assay. The data are shown as the means with SD of 6–9 fields selected at random. $^{***}p < 0.001$ vs the control. (C) MKN1 cells that had been treated with TNF-α (10 ng/ml) for 5 days were cultured for 48 h in RPMI 1640 (serum-free), and the conditioned medium was subjected to gelatin zymography. (D) The conditioned medium of MKN1 cells treated with TNF-α for 5 days was assayed for fibronectin by immunoblotting.

Discussion

In the present study, we assessed the phenotypic changes of tumor cells induced by co-culture with monocytes, and found that some tumor cells changed their phenotypes related to cell adhesion, migration and invasion. MKN1 gastric carcinoma cells and HT1080 fibrosarcoma cells acquired higher invasive potential into Matrigel basement membranes, in parallel with increased production of MMP-9 (Figs. 2-1 and 2-2). However, no other cell lines exhibited such changes after the co-culture. These phenotypic changes of tumor cells induced by monocytes thus seemed to depend on the type of tumor cells. One possible explanation is that monocytes were differentially stimulated by co-culture with distinct types of tumor cells. Because the present study suggests that TNF-α secreted from monocytes is an important factor to increase the invasive potential of tumor cells, the difference in TNF-α productivity of monocytes differentiated by various tumor cell lines may cause the differential effects on the invasive potential and MMP productivity of tumor cells. We tried to determine the TNF-α concentration in each co-culture supernatant, however, it was found to be below the detection level (<50 pg/ml). Another possibility is the differences in susceptibility of tumor cells to TNF-α. The distinct responses of tumor cells to the co-culture with monocytes observed in the present study may reflect the heterogeneity of TAMs found in clinical researches and thus cause varied prognosis of patients [47,48]. The present study indicated a correlation between the invasive potential and productivity of MMP-9 of tumor cells after co-culture with monocytes. In addition, the enhanced invasion of tumor cells was suppressed more effectively by an MMP-9 inhibitor than by an MMP-2 inhibitor. We therefore conclude that the enhanced invasion of MKN1 cells into Matrigel after the co-culture was mainly due to the increased secretion of MMP-9 from these cells.

The present study also indicated that MKN1 cell invasion potentiated by the co-culture with monocytes was suppressed in the presence of an integrin-inhibiting RGD peptide. This result strongly suggested the involvement of integrin-mediated cell adhesion in the enhanced cell invasion. It is likely that the enhanced invasion of tumor cells is mediated by the interaction between fibronectin deposited by these cells and α5β1 integrin

expressed on their surface. The enhancement of invasive potential of tumor cells was well correlated with the increased secretion of fibronectin and expression of α5 integrin (Fig. 2-5). Because the transmigration of MKN1 cells through the membranes without Matrigel coating was also potentiated by the co-culture with monocytes (unpublished observation), cell motility was likely to be promoted by the fibronectin-α5β1 integrin interaction. This interaction may also induce the morphological change of MKN1 cells after the co-culture. It has been suggested that the fibronectin-α5β1 integrin interaction activates intracellular signaling cascades including focal adhesion kinase (FAK), mitogen-activated protein kinases (MAPK), Rho GTPases, and the PI3-kinase/Akt/mTOR pathway [54,60], which may lead to stimulation of MMP-9 production and cell motility [54,58,59]. Furthermore, our previous study showed that fibronectin promoted the differentiation of monocytes into MMP-9-producing macrophages (Chapter 1), and therefore MMP-9 produced by these macrophages is also likely to facilitate tumor cell invasion.

Recently, Tsubota and co-workers [67] reported that MKN1 cells were stimulated by TNF-α to acquire higher invasive potential accompanied by changes into fibroblast-like morphology and increased vimentin expression, both of which features were similar to those observed in the process of so-called epithelial-mesenchymal transition [64,65]. They also suggested a possible relationship between enhanced secretion of a laminin component (γ2 chain) and the invasive potential of tumor cells. Because several studies have noted that the interaction of tumor cells with ECM proteins during tumor invasion and metastatic processes is greatly influenced by inflammatory cytokines [31,50], we assessed the role of TNF-α in monocyte-induced enhancement of tumor cell invasion, and found that TNF-α secreted from monocytes is, at least in part, involved in the potentiation of cell invasion.

Finally, considering our previous observation that fibronectin secreted from tumor cells promotes monocyte differentiation into MMP-9-producing macrophages (Chapter 1), we can postulate that fibronectin and MMP-9 secreted from tumor cells synergistically facilitate tumor cell invasion. The interactions between tumor cells and monocytes/macrophages mediated by ECM proteins need to be further characterized in future studies.

The person has to be educated, but we have also to educate ourselves.
There is no progress without change.

General Discussion

In the present study, I attempted to differentiate monocytes *in vitro* into TAM-like cells, and successfully induced monocytes to produce high levels of MMP-9, one of characteristic markers of TAMs, by co-culture with tumor cell lines. I then evaluated effect of TAM-like cells on *in vitro* invasion of tumor cells. The results indicated that differentiated monocytes promoted tumor cell invasion in an MMP-9-dependent manner. The MMP-9 productivity of differentiated monocytes was accompanied by macrophage-like spreading morphological changes. Therefore, I focused on cell adhesion to ECM proteins and evaluated its contribution to monocyte differentiation. The results showed that RGD (Arg-Gly-Asp)-dependent and fibronectin-mediated cell adhesion played a critical role in monocyte differentiation. Thus, I provided a useful model to investigate the process of differentiation of TAMs, and showed that cell adhesion is important for monocyte differentiation into TAMs. In Chapter 2, I assessed the phenotypic changes of tumor cells induced by co-culture with monocytes using the same model. I found that some tumor cells changed their phenotypes such as increased expression of MMP-9, fibronectin and α5 integrin, and acquired high invasive potential. Cell adhesion molecules were also shown to be important in these phenotypic changes of tumor cells. Furthermore, I presented evidence that TNF-α produced by monocytes was, at least in part, involved in the potentiation of tumor cell invasion. Finally, I can postulate that the increased fibronectin production by tumor cells synergistically promote monocyte differentiation to TAMs, and facilitate tumor invasion and angiogenesis (Chart 3).

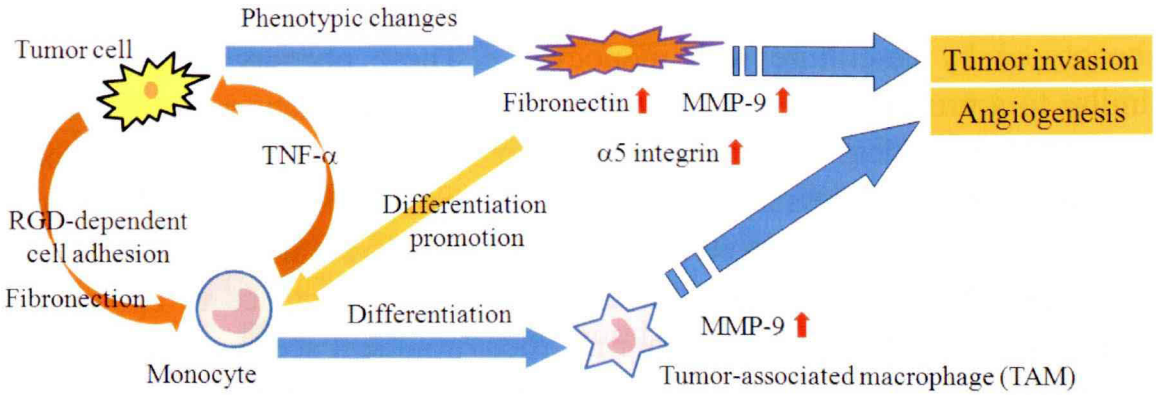

Chart 3. Schematic view of summary of this study. Tumor cells and monocytes interact through RGD-dependent and fibronectin-mediated cell adhesion. As a result, monocytes differentiate to tumor-associated macrophages (TAMs) with high MMP-9 productivity, and promote tumor invasion and angiogenesis. On the other hand, tumor cells also change their phenotypes by TNF-α produced by monocytes. Tumor invasion and angiogenesis are promoted by increased expression of fibronectin, α5 integrin and MMP-9 in tumor cells. Furthermore, fibronectin secreted from tumor cells promotes monocyte differentiation to TAMs.

In this study, I focused on TAM which is one of components of tumor microenvironment. TAMs are known as a key component of the crosstalk between tumor cells and their microenvironment. Tumor-stromal cells have been so far considered as cell population to fill the space between tumor cells. However, recent studies suggested that tumor-stromal cells affect malignant behaviors (i.e. growth, survival, invasion, metastasis and angiogenesis) of tumor cells as well as the role as simple packing cells [68,69]. For example, fibroblasts constituting tumor stroma are called cancer-associated fibroblasts (CAFs) secrete MMPs whereby promote invasion of tumor cells similar to TAMs [70,71]. In addition, endothelial cells, inflammatory cells and various other cells were infiltrated into tumor tissues [4]. These cells contribute to tumor promotion in various effects, and the interaction among these cells including tumor cells may promote tumor progression.

Loss of epithelial phenotypes and the acquisition of mesenchymal characteristics are typical for cancer cells during their progression and correlate with metastatic potential. This phenomenon is called epithelial-mesenchymal transition (EMT), and tumor cells show mesenchymal cell markers and invasive phenotypes [65,72,73]. In this study, tumor cells were shown to express

mesenchymal cell markers (i.e. fibronectin and vimentin) and invasive phenotypes by co-culture with monocytes. These phenotypic changes were similar to a property of EMT. On the other hand, the cancer stem cell (CSC) hypothesis provides an attractive cellular mechanism to account for the therapeutic refractoriness and malignant behaviors exhibited by many of tumor cells [74-76]. Parallels between the attributes of EMT and CSC were shown including enhanced metastasis, enhanced survival, resistance to apotosis, and resistance to chemotherapy. Tumor microenvironment seems to be involved in acquisition of EMT and CSC characters. Previous studies suggested that tumor microenvironment including soluble factors such as inflammatory cytokines and transforming growth factor (TGF)-β were involved in these characteristic acquisitions [75,77-79]. TNF-α secreted from TAMs changed phenotypes of tumor cells like EMT as shown in this study.

Although ECM proteins are major components in tumor microenvironment, many studies have focused on soluble factors such as cytokines. The roles of cell-to-cell and cell-to-ECM adhesion need to be further investigated. I suggested in this study that fibronectin produced by tumor cells plays a critical role in monocyte differentiation, but it is of note that neither laminin-1 nor Matrigel exerted a positive effect on this differentiation (Chapter 1). Specific interactions between ECM proteins and monocytes seem to be required for the differentiation process. It has been reported that remodeling of ECM proteins (e.g. fibronectin and laminin-5) modifies their biological activities, and ECM protein-mediated cell migration and differentiation [55,80-82]. The ECM protein remodeling is catalyzed by proteases such as MMPs. It is possible that ECM proteins in tumor tissues are remodeled because a variety of proteases including MMPs are present [83-85]. The effects of the remodeled ECM proteins and various other ECM proteins on monocyte differentiation/activation are interesting issues to be examined in the future study. Furthermore, it is also interesting to elucidate the effects of environmental conditions surrounding tumor cells and tumor-stromal cells such as hypoxia, undernutrition, acidosis and oxidative stress [86,87].

The elucidation of the tumor-microenvironment interaction is a very difficult problem, but it should be clarified the in future study. Understanding of tumor microenvironment may develop new diagnostic markers and treatment strategy of cancer.

References

1) Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL, Weaver VM, Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell, **139**, 891-906 (2009).
2) Calorini L, Bianchini F, Environmental control of invasiveness and metastatic dissemination of tumor cells: the role of tumor cell-host cell interactions. Cell Commun Signal, **8**, 24 (2010).
3) Ungefroren H, Sebens S, Seidl D, Lehnert H, Hass R, Interaction of tumor cells with the microenvironment. Cell Commun Signal, **9**, 18 (2011).
4) Castells M, Thibault B, Delord JP, Couderc B, Implication of tumor microenvironment in chemoresistance: tumor-associated stromal cells protect tumor cells from cell death. Int J Mol Sci, **13**, 9545-9571 (2012).
5) Pollard JW, Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer, **4**, 71-78 (2004).
6) Condeelis J, Pollard JW, Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell, **124**, 263-266 (2006).
7) Balkwill F, Charles KA, Mantovani A, Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell, **7**, 211-217 (2005).
8) Lewis JS, Landers RJ, Underwood JC, Harris AL, Lewis CE, Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. J Pathol, **192**, 150-158 (2000).
9) Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM, Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science, **258**, 1798-1801 (1992).
10) Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J, A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res, **64**, 7022-7029 (2004).
11) Uutela M, Wirzenius M, Paavonen K, Rajantie I, He Y, Karpanen T, Lohela M, Wiig H, Salven P, Pajusola K, Eriksson U, Alitalo K, PDGF-D induces macrophage recruitment, increased interstitial pressure, and blood vessel maturation during angiogenesis. Blood, **104**, 3198-3204 (2004).

12) Torisu H, Ono M, Kiryu H, Furue M, Ohmoto Y, Nakayama J, Nishioka Y, Sone S, Kuwano M, Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFalpha and IL-1alpha. Int J Cancer, **85**, 182-188 (2000).
13) Hagemann T, Robinson SC, Schulz M, Trumper L, Balkwill FR, Binder C, Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases. Carcinogenesis, **25**, 1543-1549 (2004).
14) Marconi C, Bianchini F, Mannini A, Mugnai G, Ruggieri S, Calorini L, Tumoral and macrophage uPAR and MMP-9 contribute to the invasiveness of B16 murine melanoma cells. Clin Exp Metastasis, **25**, 225-231 (2008).
15) Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL, Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res, **56**, 4625-4629 (1996).
16) Bingle L, Brown NJ, Lewis CE, The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol, **196**, 254-265 (2002).
17) Lewis CE, Pollard JW, Distinct role of macrophages in different tumor microenvironments. Cancer Res, **66**, 605-612 (2006).
18) Graves DT, Jiang YL, Williamson MJ, Valente AJ, Identification of monocyte chemotactic activity produced by malignant cells. Science, **245**, 1490-1493 (1989).
19) Muramatsu M, Yamamoto S, Osawa T, Shibuya M, Vascular endothelial growth factor receptor-1 signaling promotes mobilization of macrophage lineage cells from bone marrow and stimulates solid tumor growth. Cancer Res, **70**, 8211-8221 (2010).
20) Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M, The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol, **25**, 677-686 (2004).
21) Mantovani A, Sozzani S, Locati M, Allavena P, Sica A, Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol, **23**, 549-555 (2002).
22) Solinas G, Germano G, Mantovani A, Allavena P, Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol, **86**, 1065-1073 (2009).
23) Hagemann T, Wilson J, Burke F, Kulbe H, Li NF, Pluddemann A, Charles K, Gordon S, Balkwill FR, Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype. J Immunol, **176**, 5023-5032 (2006).

24) Solinas G, Schiarea S, Liguori M, Fabbri M, Pesce S, Zammataro L, Pasqualini F, Nebuloni M, Chiabrando C, Mantovani A, Allavena P, Tumor-conditioned macrophages secrete migration-stimulating factor: a new marker for M2-polarization, influencing tumor cell motility. J Immunol, **185**, 642-652 (2010).

25) Lin EY, Nguyen AV, Russell RG, Pollard JW, Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med, **193**, 727-740 (2001).

26) Sica A, Saccani A, Bottazzi B, Polentarutti N, Vecchi A, van Damme J, Mantovani A, Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. J Immunol, **164**, 762-767 (2000).

27) Duluc D, Delneste Y, Tan F, Moles MP, Grimaud L, Lenoir J, Preisser L, Anegon I, Catala L, Ifrah N, Descamps P, Gamelin E, Gascan H, Hebbar M, Jeannin P, Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. Blood, **110**, 4319-4330 (2007).

28) Hagemann T, Lawrence T, McNeish I, Charles KA, Kulbe H, Thompson RG, Robinson SC, Balkwill FR, "Re-educating" tumor-associated macrophages by targeting NF-kappaB. J Exp Med, **205**, 1261-1268 (2008).

29) Hagemann T, Biswas SK, Lawrence T, Sica A, Lewis CE, Regulation of macrophage function in tumors: the multifaceted role of NF-kappaB. Blood, **113**, 3139-3146 (2009).

30) Luo JL, Maeda S, Hsu LC, Yagita H, Karin M, Inhibition of NF-kappaB in cancer cells converts inflammation- induced tumor growth mediated by TNFalpha to TRAIL-mediated tumor regression. Cancer Cell, **6**, 297-305 (2004).

31) Li Q, Withoff S, Verma IM, Inflammation-associated cancer: NF-kappaB is the lynchpin. Trends Immunol, **26**, 318-325 (2005).

32) Barczyk M, Carracedo S, Gullberg D, Integrins. Cell Tissue Res, **339**, 269-280 (2010).

33) Giancotti FG, Ruoslahti E, Integrin signaling. Science, **285**, 1028-1032 (1999).

34) Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM, M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol, **164**, 6166-6173 (2000).

35) Dirkx AE, Oude Egbrink MG, Wagstaff J, Griffioen AW, Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. J Leukoc Biol, **80**, 1183-1196 (2006).

36) Sudhakaran PR, Radhika A, Jacob SS, Monocyte macrophage differentiation in vitro: Fibronectin-dependent upregulation of certain macrophage-specific activities. Glycoconj J, **24**, 49-55 (2007).
37) Terui Y, Furukawa Y, Sakai T, Kikuchi J, Sugahara H, Kanakura Y, Kitagawa S, Miura Y, Up-regulation of VLA-5 expression during monocytic differentiation and its role in negative control of the survival of peripheral blood monocytes. J Immunol, **156**, 1981-1988 (1996).
38) Koike J, Nagata K, Kudo S, Tsuji T, Irimura T, Density-dependent induction of TNF-alpha release from human monocytes by immobilized P-selectin. FEBS Lett, **477**, 84-88 (2000).
39) Itoh S, Hamada E, Kamoshida G, Takeshita K, Oku T, Tsuji T, Staphylococcal superantigen-like protein 5 inhibits matrix metalloproteinase 9 from human neutrophils. Infect Immun, **78**, 3298-3305 (2010).
40) Tsuji T, Kawada Y, Kai-Murozono M, Komatsu S, Han SA, Takeuchi K, Mizushima H, Miyazaki K, Irimura T, Regulation of melanoma cell migration and invasion by laminin-5 and alpha3beta1 integrin (VLA-3). Clin Exp Metastasis, **19**, 127-134 (2002).
41) Chomczynski P, Sacchi N, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem, **162**, 156-159 (1987).
42) Katabami K, Mizuno H, Sano R, Saito Y, Ogura M, Itoh S, Tsuji T, Transforming growth factor-beta1 upregulates transcription of alpha3 integrin gene in hepatocellular carcinoma cells via Ets-transcription factor-binding motif in the promoter region. Clin Exp Metastasis, **22**, 539-548 (2005).
43) Takeuchi K, Tsuji T, Hakomori S, Irimura T, Intercellular adhesion induced by anti-alpha 3 integrin (VLA-3) antibodies. Exp Cell Res, **211**, 133-141 (1994).
44) Tsuji T, Physiological and pathological roles of alpha3beta1 integrin. J Membr Biol, **200**, 115-132 (2004).
45) Goodman SL, Aumailley M, von der Mark H, Multiple cell surface receptors for the short arms of laminin: alpha 1 beta 1 integrin and RGD-dependent proteins mediate cell attachment only to domains III in murine tumor laminin. J Cell Biol, **113**, 931-941 (1991).
46) Pierschbacher MD, Ruoslahti E, Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature, **309**, 30-33 (1984).

47) Brigati C, Noonan DM, Albini A, Benelli R, Tumors and inflammatory infiltrates: friends or foes? Clin Exp Metastasis, **19**, 247-258 (2002).
48) de Visser KE, Eichten A, Coussens LM, Paradoxical roles of the immune system during cancer development. Nat Rev Cancer, **6**, 24-37 (2006).
49) Lin CY, Lin CJ, Chen KH, Wu JC, Huang SH, Wang SM, Macrophage activation increases the invasive properties of hepatoma cells by destabilization of the adherens junction. FEBS Lett, **580**, 3042-3050 (2006).
50) Liotta LA, Tumor invasion and metastases--role of the extracellular matrix: Rhoads Memorial Award lecture. Cancer Res, **46**, 1-7 (1986).
51) Johansson N, Ahonen M, Kahari VM, Matrix metalloproteinases in tumor invasion. Cell Mol Life Sci, **57**, 5-15 (2000).
52) Egeblad M, Werb Z, New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer, **2**, 161-174 (2002).
53) Akiyama SK, Olden K, Yamada KM, Fibronectin and integrins in invasion and metastasis. Cancer Metastasis Rev, **14**, 173-189 (1995).
54) Ritzenthaler JD, Han S, Roman J, Stimulation of lung carcinoma cell growth by fibronectin-integrin signalling. Mol Biosyst, **4**, 1160-1169 (2008).
55) Miyazaki K, Laminin-5 (laminin-332): Unique biological activity and role in tumor growth and invasion. Cancer Sci, **97**, 91-98 (2006).
56) Hancox RA, Allen MD, Holliday DL, Edwards DR, Pennington CJ, Guttery DS, Shaw JA, Walker RA, Pringle JH, Jones JL, Tumour-associated tenascin-C isoforms promote breast cancer cell invasion and growth by matrix metalloproteinase-dependent and independent mechanisms. Breast Cancer Res, **11**, R24 (2009).
57) Saito Y, Sekine W, Sano R, Komatsu S, Mizuno H, Katabami K, Shimada K, Oku T, Tsuji T, Potentiation of cell invasion and matrix metalloproteinase production by alpha3beta1 integrin-mediated adhesion of gastric carcinoma cells to laminin-5. Clin Exp Metastasis, **27**, 197-205 (2010).
58) Wei Y, Tang CH, Kim Y, Robillard L, Zhang F, Kugler MC, Chapman HA, Urokinase receptors are required for alpha 5 beta 1 integrin-mediated signaling in tumor cells. J Biol Chem, **282**, 3929-3939 (2007).
59) Thant AA, Nawa A, Kikkawa F, Ichigotani Y, Zhang Y, Sein TT, Amin AR, Hamaguchi M, Fibronectin activates matrix metalloproteinase-9 secretion via the MEK1-MAPK and the PI3K-Akt pathways in ovarian cancer cells. Clin Exp Metastasis, **18**, 423-428 (2000).
60) Kornberg LJ, Earp HS, Turner CE, Prockop C, Juliano RL, Signal transduction by integrins: increased protein tyrosine phosphorylation

caused by clustering of beta 1 integrins. Proc Natl Acad Sci U S A, **88**, 8392-8396 (1991).

61) Qian F, Zhang ZC, Wu XF, Li YP, Xu Q, Interaction between integrin alpha(5) and fibronectin is required for metastasis of B16F10 melanoma cells. Biochem Biophys Res Commun, **333**, 1269-1275 (2005).

62) Zheng M, Fang H, Tsuruoka T, Tsuji T, Sasaki T, Hakomori S, Regulatory role of GM3 ganglioside in alpha 5 beta 1 integrin receptor for fibronectin-mediated adhesion of FUA169 cells. J Biol Chem, **268**, 2217-2222 (1993).

63) Nagaharu K, Zhang X, Yoshida T, Katoh D, Hanamura N, Kozuka Y, Ogawa T, Shiraishi T, Imanaka-Yoshida K, Tenascin C induces epithelial-mesenchymal transition-like change accompanied by SRC activation and focal adhesion kinase phosphorylation in human breast cancer cells. Am J Pathol, **178**, 754-763 (2011).

64) Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA, The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell, **133**, 704-715 (2008).

65) Thiery JP, Acloque H, Huang RY, Nieto MA, Epithelial-mesenchymal transitions in development and disease. Cell, **139**, 871-890 (2009).

66) Karin M, Nuclear factor-kappaB in cancer development and progression. Nature, **441**, 431-436 (2006).

67) Tsubota Y, Ogawa T, Oyanagi J, Nagashima Y, Miyazaki K, Expression of laminin gamma2 chain monomer enhances invasive growth of human carcinoma cells in vivo. Int J Cancer, **127**, 2031-2041 (2010).

68) Li H, Fan X, Houghton J, Tumor microenvironment: the role of the tumor stroma in cancer. J Cell Biochem, **101**, 805-815 (2007).

69) Liotta LA, Kohn EC, The microenvironment of the tumour-host interface. Nature, **411**, 375-379 (2001).

70) Giannoni E, Bianchini F, Masieri L, Serni S, Torre E, Calorini L, Chiarugi P, Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. Cancer Res, **70**, 6945-6956 (2010).

71) Bhowmick NA, Neilson EG, Moses HL, Stromal fibroblasts in cancer initiation and progression. Nature, **432**, 332-337 (2004).

72) Zavadil J, Haley J, Kalluri R, Muthuswamy SK, Thompson E, Epithelial-mesenchymal transition. Cancer Res, **68**, 9574-9577 (2008).

73) Thiery JP, Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer, **2**, 442-454 (2002).
74) Gupta PB, Chaffer CL, Weinberg RA, Cancer stem cells: mirage or reality? Nat Med, **15**, 1010-1012 (2009).
75) Visvader JE, Lindeman GJ, Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer, **8**, 755-768 (2008).
76) Dalerba P, Cho RW, Clarke MF, Cancer stem cells: models and concepts. Annu Rev Med, **58**, 267-284 (2007).
77) Santisteban M, Reiman JM, Asiedu MK, Behrens MD, Nassar A, Kalli KR, Haluska P, Ingle JN, Hartmann LC, Manjili MH, Radisky DC, Ferrone S, Knutson KL, Immune-induced epithelial to mesenchymal transition in vivo generates breast cancer stem cells. Cancer Res, **69**, 2887-2895 (2009).
78) Shimizu T, Ishikawa T, Iwai S, Ueki A, Sugihara E, Onishi N, Kuninaka S, Miyamoto T, Toyama Y, Ijiri H, Mori H, Matsuzaki Y, Yaguchi T, Nishio H, Kawakami Y, Ikeda Y, Saya H, Fibroblast growth factor-2 is an important factor that maintains cellular immaturity and contributes to aggressiveness of osteosarcoma. Mol Cancer Res, **10**, 454-468 (2012).
79) Tse JC, Kalluri R, Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. J Cell Biochem, **101**, 816-829 (2007).
80) Lagana A, Goetz JG, Cheung P, Raz A, Dennis JW, Nabi IR, Galectin binding to Mgat5-modified N-glycans regulates fibronectin matrix remodeling in tumor cells. Mol Cell Biol, **26**, 3181-3193 (2006).
81) Ogawa T, Tsubota Y, Maeda M, Kariya Y, Miyazaki K, Regulation of biological activity of laminin-5 by proteolytic processing of gamma2 chain. J Cell Biochem, **92**, 701-714 (2004).
82) Buzza MS, Zamurs L, Sun J, Bird CH, Smith AI, Trapani JA, Froelich CJ, Nice EC, Bird PI, Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin. J Biol Chem, **280**, 23549-23558 (2005).
83) DeClerck YA, Mercurio AM, Stack MS, Chapman HA, Zutter MM, Muschel RJ, Raz A, Matrisian LM, Sloane BF, Noel A, Hendrix MJ, Coussens L, Padarathsingh M, Proteases, extracellular matrix, and cancer: a workshop of the path B study section. Am J Pathol, **164**, 1131-1139 (2004).
84) Bissell MJ, Kenny PA, Radisky DC, Microenvironmental regulators of tissue structure and function also regulate tumor induction and

progression: the role of extracellular matrix and its degrading enzymes. Cold Spring Harb Symp Quant Biol, **70**, 343-356 (2005).

85) Kessenbrock K, Plaks V, Werb Z, Matrix metalloproteinases: regulators of the tumor microenvironment. Cell, **141**, 52-67 (2010).

86) Hockel M, Vaupel P, Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst, **93**, 266-276 (2001).

87) Tredan O, Galmarini CM, Patel K, Tannock IF, Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst, **99**, 1441-1454 (2007).

Acknowledgements

本研究を行うにあたりまして，終始温かく多大な御支援と御指導，御鞭撻を賜り，また本論文の御校閲を頂きました星薬科大学 微生物学教室 辻 勉 教授に謹んで厚く御礼申し上げます.

本研究を推進するにあたり，御指導，御鞭撻を賜りました東京大学薬学部 生体異物学教室 入村 達郎 教授に謹んで御礼申し上げます.

本研究を行うにあたり，多大な御指導，御協力を賜りました名古屋市立大学薬学部 生体防御機能学教室 小野嵜 菊夫 教授，瀧井 猛将 准教授，伊藤 佐生智 講師に厚く御礼申し上げます. 特に，伊藤 佐生智 講師には兄のように接して頂き，昼夜を問わず数々の場面で有益な御助言，御協力を頂きました. 重ねて御礼申し上げます.

本研究を推進するにあたり，温かく多大な御指導，御助言を賜りました横浜市立大学木原生物学研究所 細胞生物学部門 宮崎 香 教授，東 昌市 准教授，古宮 栄利子 博士をはじめとする教室の皆様に深く感謝申し上げます.

本研究を行うにあたり，御助言，御協力を賜りました星薬科大学 病態機能制御学研究室 高橋 典子 教授，星薬科大学 創剤構築研究室 米谷 芳枝 教授，服部 喜之 准教授，医療法人社団 栗原医院 栗原 正明 医師に深く御礼申し上げます.

また，星薬科大学 微生物学教室の皆様には約6年もの間，大変お世話になり，深く感謝申し上げます. さらに，水野 博己氏，関根 わか菜氏をはじめとする先輩の方々にも公私共にお世話になり，重ねて御礼申し上げます.

そして，本研究を行うにあたり，協力を頂き，公私共々支えて頂きました 松田 彩花氏，中村 雄一氏，高橋 泉恵氏，桂 有沙氏，高嶋 友理氏，三浦 莉紗氏，鈴木 沙由里氏，石和 迪朗氏，西村 友吾氏，宮嶋 崇樹氏，板岡 愛美氏，古川 歩氏，森 久根氏，Enric Llorens 氏には深く感謝申し上げます. 特に，松田 彩花氏には数々の場面で助言，協力を頂き，重ねて心から感謝致します.

最後に，本研究を行える環境を与えて頂き，あらゆる面で支援してくれた両親，そして，これまで私を支えてくれたすべての方々に心より感謝致します.

Every person's work is a nature of oneself.
When work is a pleasure, life is beautiful. When work is a duty, life is slavery.
That means, I pride what I am doing, believe myself most, and will become special one.
You know what I mean.