博 士（医 学） ダルマニン ステファニー

学 位 論 文 題 名

All-*trans* Retinoic Acid Enhances Murine Dendritic Cell Migration to Draining Lymph Nodes via the Balance of Matrix Metalloproteinases and Their Inhibitors

（All-*trans* retinoic acid は MMP と MMP 阻害因子の産生制御を介して、マウス樹状細胞の所属リンパ節への遊走を増強する）

学位論文内容の要旨

Background and Aim

Cancers escape immune surveillance through the manipulation of the host's immune system. Sequestration of dendritic cells (DCs) within tumor tissues and the subsequent inhibition of their migration is one of the several mechanisms by which tumors induce immunosuppression. In view of recent findings, depicting the improvement of tumor immune responses in cancer patients following treatment with the vitamin A derivative, all-*trans* retinoic acid (ATRA), we sought to identify the effects of ATRA on DC mobility in the context of tumor immunotherapy.

Materials and Methods

DCs were obtained by culturing C57BL/6 murine bone marrow progenitor cells in the presence of GM-CSF and IL-4 for 8 days, with or without ATRA (1μM), and where appropriate, DCs were matured with LPS (1μg/ml) for a further 24 hrs. Flow cytometry was used to analyze the surface expression of the DC marker CD11c, CCR7, MHCII and the co-stimulatory molecules CD40, CD80 and CD86, as well as the phagocytosis of FITC-dextran by the DCs. *In vitro* migration of these cells was confirmed by means of Transwell® chamber assays, in the absence or presence of extracellular matrix (ECM), the recombinant murine chemokines CCL19 and CCL21, and the broad-spectrum MMP inhibitor GM6001. To monitor *in vivo* migration, immature DCs (radioactively labeled with ^{111}In or ^{51}Cr) were injected in a palpable B16BL/6 tumor already present in the right flank of mice. Scintigraphic imaging with a gamma camera after 24 hrs depicted the accumulation of ^{111}In within the mice. After 48 hrs the radioactive emissions from the draining lymph nodes, non-draining lymph nodes and spleens were obtained. These were expressed as a percentage of the radioactive emissions from the initially injected cells, samples of which were stored at 4°C for 48 hrs. Real-time PCR was used to confirm the mRNA expression levels of CCR-7, the matrix metalloproteinases (MMPs) MMP-9, MMP-14 and MMP-2 and their inhibitors TIMP-1, TIMP-2 and TIMP-3 in the DCs. Also, the enzymatic activity of MMP-9 and MMP-2 in DC culture supernatants was analyzed by gelatin zymography. Statistical analyses were performed on all results obtained, and a p-value ≤ 0.05 was considered significant.

Results

The immunophenotype and phagocytic capacity of the DCs generated in the presence of

ATRA were comparable to those of their untreated counterparts, confirming that these cells have an adequate phenotype for their function. Results obtained from migration experiments demonstrate that ATRA, added to differentiating murine bone marrow progenitor cells, enhances the invasive capacity of the resulting DCs. The *in vitro* migration of mature DCs through ECM, towards the lymphoid chemokines, CCL19 and CCL21, is enhanced when ATRA is present during their differentiation. Moreover, when immature DCs are injected intra-tumorally in mice, they show increased accumulation in draining lymph nodes, but not in non-draining lymph nodes and spleens, when differentiated in the presence of ATRA. Interestingly, both CCR7 mRNA and surface expression were not significantly altered by ATRA in the DCs. An increase in MMP production with a simultaneous decrease in the production of their inhibitors (TIMPs), however, is provoked by ATRA. This affects the MMP/TIMP balance in DCs, in particular that of MMP-9 and TIMP-1, favoring protease activity and thus allowing for enhanced DC mobilization. The role of MMPs in the ATRA-mediated increased DC migration was confirmed by means of a broad-spectrum MMP inhibitor, which completely inhibited the effects of ATRA, thereby ascertaining that the increase in DC migration brought about by ATRA is, in fact, dependent on MMPs.

Discussion

Cancer immunotherapy is a field of intense research aiming to induce active immunity with either therapeutic or adjuvant intent. DCs, being the most potent antigen-presenting cells (APCs) of the immune system, represent a promising tool in therapeutic vaccination against cancer. Clinical trials have indicated that DC vaccines are feasible and safe with minimal side effects, and effective in some patients, particularly if the DCs have been appropriately matured and activated. Hence, numerous immunization strategies involving DCs are in various stages of investigation. Despite the success obtained to date in animal studies, however, and to a lesser extent in clinical trials, manipulation of the migration of *ex vivo*-generated DCs from the site of injection to the lymph nodes remains a major challenge in this field. As a result, the notion of *in situ* targeting of DCs and thus the use of the efficient migratory properties of DCs *in vivo* is gaining popularity. Among the attempts to target DCs *in situ* and at the same time provide appropriate maturation and activation stimuli the use of a synthetic CpG oligonucleotide, which is known to trigger Toll-like receptor 9 (TLR9), to immunize melanoma patients with a Melan-A peptide in a clinical trial yielded particularly successful results. Thanks to such work it is now established that there is great promise in the use of chemical adjuvants, both in combination with cancer vaccines, as well as to elicit anti-tumor immune responses in general.

As a naturally-occurring isomer of retinoic acid, ATRA is a well known factor capable to induce the differentiation of acute promyelocytic leukemia (APL) cells, and was thus successfully utilized in the treatment of APL patients. The current study demonstrates that ATRA is capable of improving DC trafficking in a tumor milieu, confirming that the modulation of the protease/anti-protease balance by ATRA may have an impact on disease pathogenesis, and that moreover, this characteristic of ATRA can be exploited in DCs, thus making ATRA an attractive candidate for use as a chemical adjuvant within the cancer scenario.

Conclusion

Considering the implications of DC migration in a tumor milieu, ATRA might conceivably be a valuable addition both to cancer vaccines as well as to cancer immunotherapy overall. In conclusion, our findings, together with the encouraging results obtained in the clinic, support the rationale for integrating ATRA in the standard therapeutic arsenal used in cancer patient care.

学位論文審査の要旨

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癌細胞は宿主の免疫機構を修飾することで免疫監視機構から逃避するとされており、樹状細胞の腫瘍組織から所属リンパ節への移動阻害は腫瘍が誘導する免疫抑制機構のメカニズムのひとつと考えられる。ATRA によるがん患者の免疫応答改善に関する最近の報告と、ATRA による APL 細胞の運動能増強に関する報告とを合わせると、ATRA によって樹状細胞の運動能改善を介して免疫抑制が解除される可能性が期待され、申請者は ATRA の樹状細胞運動能に与える影響を検討した。その結果、マウス骨髄細胞からの樹状細胞樹立過程に ATRA を添加すると、以下のように樹状細胞の浸潤能増強が認められた。ATRA 処理樹状細胞を腫瘍内に注入すると、所属リンパ節への集積が増強した。さらに、ATRA 処理樹状細胞のケモカイン CCL19 および CCL21 に対する浸潤能が増強した。この浸潤能の増強は MMP 阻害因子の存在下では消失した。ATRA で処理すると、樹状細胞の MMP 産生が亢進し、MMP 阻害因子の産生が抑制された。これらの結果は、ATRA 処理によって MMP/TIMP バランスがプロテアーゼ活性に傾いて樹状細胞の浸潤能を増強させること、さらに ATRA 処理によって樹状細胞の免疫応答機能が増強される可能性を示唆している。

口頭発表に際し、副査の岩渕和也准教授より樹状細胞はどのタイプのレチノイン酸レセプターを発現しているのか、MMP-9 と TIMP-1 の遺伝子上流にはレチノイン酸レセプターの結合部位があるのか、骨髄由来以外の樹状細胞において同様の効果が得られるのか、担癌マウスでの抗腫瘍効果は認められるのか、との質問があった。これに対して申請者は、骨髄由来の樹状細胞では RARα が発現していること、MMP-9 と TIMP-1 が ATRA の標的遺伝子と報告されていること、今回の検討で直接調べていない他のタイプの樹状細胞に関しては今後調べる必要があること、肺転移の低下傾向が認められたことから、T 細胞の抗腫瘍活性の増強まで得られた可能性が考えられると回答した。副査の守内哲也教授より腫瘍

に対するT細胞応答をみているのか、腫瘍抗原を添加することによって免疫応答を改善できるのか、さらに、樹状細胞へのMMP-9遺伝子導入によって樹状細胞の運動能増強が見られるのかについて質問があった。これに対し申請者は、肺転移の抑制などT細胞機能が増強した可能性が示唆されると回答し、また正常細胞である樹状細胞への遺伝子導入実験は技術的な難しさゆえに行っていないと回答した。最後に主査の今村雅寛教授から、今回の研究をどのように臨床応用していくのか、腫瘍の再チャレンジによるメモリーT細胞の存在の検討を行ったのか、また他の腫瘍系での検討を行ったのか、との質問があった。これに対し申請者は、樹状細胞免疫療法にATRAを添加して樹状細胞を樹立するという方法を組み合わせる臨床応用が可能ではないかと回答した。メモリーT細胞と他の腫瘍系については検討していないと回答した。

本研究はATRA処理によって樹状細胞の浸潤能が増強して抗腫瘍免疫応答の改善が得られる可能性を示したことが高く評価され、今後ATRAによるMMP-9産生の亢進、TIMP-1産生低下のメカニズムがさらに解明されることと、樹状細胞を用いた免疫細胞療法への応用が期待される。

審査員一同は、これらの成果を高く評価し、大学院課程における研鑽や取得単位なども併せ申請者が博士（医学）の学位を受けるのに充分な資格を有するものと判定した。