論 文 の 内 容 の 要 旨

論文題目「Anti-Tn-antigen MLS128 monoclonal antibody:
Its effects on colon cancer cells and characterization
and identification of its receptor」
(抗Tn抗原 MLS128抗体: 大腸がんに対するその効果とその受容体に関する研究)

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キーワード：抗Tn抗原モノクローナル抗体、大腸がん細胞、がん関連糖鎖、
細胞増殖抑制、抗体医薬

A common O-linked glycosylation with N-acetylgalactosamine (GalNAc) occurs at serine or threonine residues of glycoproteins, including mucins. GalNAcα-Ser/Thr, which is known as Tn antigen, is the precursor for all mucin-type O-glycans. Tn-antigens are shielded by extended glycosylation in healthy and benign tissues but are uncovered in approximately 90 % of carcinomas. This makes the Tn-antigen as one of the most cancer specific biomarker and potential target in cancer therapeutics. Anti-Tn antigen MLS128 monoclonal antibody (mAb) was produced two decades ago by immunizing mice with "cancerous antigens" derived from LS180 colon cancer cells.

Previous studies in our laboratory revealed that MLS128 inhibits cancer cell growth, and suggested insulin-like growth factor-I receptor (IGF-IR) down-regulation as a possible mechanism underlying the effects of MLS128. Thus, anti-IGF-IR mAb 1H7 was used in the first part of this study along with MLS128 to understand the inhibitory effect of MLS128 and to characterize its possible interactions with IGF-IR signaling in colon cancer cells. The effects of MLS128 and 1H7 mAbs on three colon cancer lines; HT29, LS180 and LS174T, were investigated. Both mAbs inhibited the growth of all three cancer cell lines examined to a certain degree. Growth inhibition by 1H7 was more significant than MLS128, but only to 60% level although it was confirmed that colon cancer cell growth is IGF-I dependent. These results suggested that there must be other growth signaling pathways involved in colon cancer cell growth other than IGF-IR signaling. This study revealed that down-regulation of the IGF-IR, which was clearly observed with those cells treated with 1H7, was responsible for the inhibition of colon cancer cell growth. In contrast, MLS128 did not down-regulate IGF-IR in colon cancer cell lines. This result ruled out the original hypothesis that the down-

regulation of IGF-IR is one possible mechanism by which MLS128 inhibits LS180 cell growth

Cumulative data suggested that MLS128 binds to a 110 kDa glycoprotein (GP) on the surface of colon cancer cells. MLS128 treatment caused a partial disappearance of 110 kDa GP on all three cancer cell lines. The disappearance of 110 kDa GP was most significantly observed in HT29 cells. Although IGF-IR down-regulation may not be the mechanism underlying MS128 inhibitory effects as proposed previously, a possible link between IGF-IR- and 110kDa GP-mediated growth signaling pathways in HT29 cells was examined by immunoprecipitation/immunoblotting (IP/IB) experiments, which did not indicate direct association of the two molecules.

The second part of this research was carried out to characterize and identify the 110 kDa GP. Microdomains are thought to consist of a wide array of proteins, lipids, receptors as well as many signaling molecules. Sucrose gradient fractionation of HT29 and LS180 cell lysates was conducted to determine whether or not IGF-IR and 110 kDa GP exist in microdomains. IB results showed that both molecules were localized in microdomains. MLS128 treatment of cells resulted in the reduction in IGF-IR and 110 kDa GP as well as Src family kinases in the microdomains. This suggested that MLS128 may have modulated microdomains, which may influence colon cancer cell growth.

Two dimensional gel electrophoresis and mass spectrometry of isolated tryptic peptides were performed to identify 110 kDa GP according to highly sophisticated conventional procedures, but so far no candidate GP has been found. This may be due to the fact that the 110 kDa GP is extremely resistant to trypsin digestion, as a result of interference by abundant O-glycosylation, the lack of database for glycosylated peptides and the limited availability of samples.

During this study, however, an interesting observation was made, that is, limited proteolysis occurred in 110 kDa GP localized in microdomains during freezing and storage. Two independent observations involving HT29 and LS180 cells suggested the existence of possibly different protease-susceptible sites in the respective 110 kDa GPs. Conformational relaxation during storage, freezing, and thawing must have exposed the cleavage site(s) to contaminating proteases, resulting in limited proteolysis as was observed. Although protease inhibitor cocktails were added to cell lysates and during sucrose gradient fractionation, degradation of 110 kDa still occurred. This fact suggests that unidentified proteases that are resistant to the added inhibitors are responsible for the limited proteolysis of 110 kDa GP. The unexpected observation of limited proteolysis should be useful for further investigation, specifically to identify 110 kDa from databases and eventually uncover its structure and the mechanisms of cell growth inhibition by MLS128.

In conclusion, with respect to the hypothesis proposed at the beginning of this study, it is now possible to state that IGF-IR down-regulation and signaling is not the mechanism involved in colon cancer cell growth inhibition by MLS128.

Significant findings from this study are that colon cancer cell growth is IGF-I dependent and that the down-regulation by anti-IGF-IR is the mechanism by which anti-IGF-IR mAb inhibits the growth of colon cancer cells. To determine the effects of MLS128 on colon cancer cell growth, identification of 110 kDa GP is inevitably required. This research has provided with hints and insights into future studies for achieving our goal.

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ムチン型糖鎖である0-結合型糖鎖はコア構造がSer/Thr残基にNアセチルガラクトサミン(GalNAc)が結合したTn抗原(GalNAca-Ser/Thr)にオリゴ糖が伸長したものであり、がん化によりTn抗原はむき出しになることからがん特異的のマーカーとして有名である。Tn抗原結合性MLS128モノクローナル抗体は20年以上前に大腸がん細胞LS180のムチンで免疫したマウスから単離された。
先行研究からMLS128処理でがん細胞増殖阻害が起こること、その阻害にはインスリン様増殖因子I受容体(IGF-IR)のダウンレギュレーションが関与している可能性が示唆されていた。そこで本研究では、3種の大腸がん細胞株を使い、第1部としてIGF-IRに対するモノクローナル抗体1H7とMLS128のがん細胞増殖への影響を調べた。その結果両方の抗体で細胞増殖阻害が見られたが、1H7による阻害効果がMLS128より大きかったこと、3種の大腸がん細胞株の増殖がIGF-I依存性であること、1H7による細胞増殖阻害はIGF-IRのダウンレギュレーションによることを明らかにした。しかし、MLS128処理でIGF-IRのダウンレギュレーションは観察されなかったことから、先に立てた仮説はほぼ否定された。

先行研究と本研究からMLS128が大腸がん細胞膜上の110 kDa glycoprotein(GP)を認識することが示唆された。MLS128処理した細胞から110 kDa GPが有意に消失こと を見出した。この効果が最も顕著に見られるHT29細胞で、免疫沈降・イムノブロット(IP/IB)実験を行ったところ、110 kDa GPとIGF-IRの直接の結合は見られなかった。

第2部の研究として、110 kDa GPの解析と同定を目指した。マイクロドメインは脂質・タンパク質・受容体のほか様々なシグナル分子が集まった細胞膜上の島のようなものと考えられている。110 kDa GPとIGF-IRが、マイクロドメインに局在すること、MLS128で処理したHT29とLS180細胞では、両分子がSrcファミリーキナーゼとともに減少することを見出した。これはMLS128がマイクロドメインに変化を与えることで細胞増殖に影響を及ぼす可能性を示唆した。

110 kDa GPの同定に向けて、2次元電気泳動と最新技術によるトリプシンペプチドのマス解析から得られたアミノ酸配列をデータベースサーチしたが、共雑物の陰に隠れて、110 kDa GPの正体を決めるまでには至らなかった。最大の理由は、0-結合型糖鎖が多数タンパク質バックボーンに付いていると予想されることから、トリプシンで切れ難く回収率が極端に低かったと考えられる。

しかしながら、これらの研究中に予想していなかった現象を見出した。それは凍結

保存中にmicrodomainに局在している110 kDa GPの限定分解が起こったことを見出したというものである。これは、3-4年間の凍結保存と融解により、110 kDa GP の高次構造変化し共雑している細胞由来のタンパク質分解酵素により分解されコア―部分が残ったと考えられる。HT29細胞とLS180細胞由来の110 kDa GPに分解後の断片サイズに違いがあること、予想されるタンパク質分解酵素のアミノ酸配列特異性から、今後110 kDa GPの同定にこの情報を有効に活用できると考えられる。

以上をまとめると、本研究の初めに立てた作業仮説であるMLS128による大腸がん細胞増殖阻害はIGF-IRのダウンレギュレーションが関与しているかは否定的結果を得た。しかし、3種の樹立株において、大腸がん細胞増殖がIGF-Iに依存すること、抗IGF-IR処理でIGF-IRがダウンレギュレーションを介して大腸がん細胞の増殖阻害をすることを明らかにした。さらに、MLS128の認識する110 kDa GPの同定に向けての新規の情報を得たことから、本研究が今後のMLS128による大腸がん細胞増殖阻害機構の解明に寄与すると考えられる。