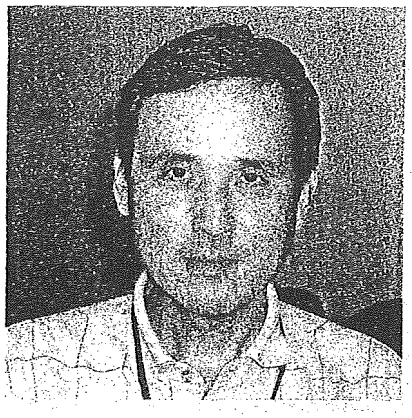

檜山行雄氏
(国立医薬品食品衛生研究所　薬品部第三室　室長)

【まず，これまでのICHでの，品質分野の成果を教えてください。】

ICHでは品質関連について，安定性試験ガイドライン(Q１)，不純物に関するガイドライン(Q３)，規格及び試験方法のガイドライン(Q６)など申請資料にどういうデータを入れるべきかという点について，国際的な調和を図ってきました。数年前からはCTD(Common Technical Document)の議論に移り，すでに申請書類のフォーマットの統一化が図られました。

【今回のICH６ではどのような動きがあったのですか。】

今回のICH本会議では，CTD，改定されたQ１，Q３の報告が行われました。またCTDの運用を踏まえて，新たに，製剤設計，製造法設計の重要性が指摘されました。

背景には，CTDの品質の部分にはPharmaceutical Development(以下，PD)という項目，つまり製剤開発についての記述の仕方が，日米欧で相当大きく異なっているのではないかという認識があります。記述の仕方だけでなく，製剤設計のステップ，内容も異なるのではないかと。そのため，製剤開発，製造法設計の仕方に話を進めなくてはならないというニーズが出てきたのです。

今年７月にベルギーで行われたICHステアリングコミッティーで，PDを品質の新しいトピックとして採用することが決まり，本会議開始直前の専門家会議でPDのガイドライン案の作成に着手されました。

【PDのガイドライン作成の意義を教えてください。】

製剤開発のステップを記述していくことによって，企業側も審査する行政側も，その情報を有効に使うことができるようになります。かつては，とくに日本では規格試験の結果をみて品質を評価する色彩の方が強かったのですが，設計の仕方をみて品質を確保しようという方向に変わります。製剤設計そのものが品質に大きく影響することは当たり前で，本来，設計段階でどうすればうまくいくかということを把握していなければ，品質管理のしようがありません。

品質とは要するに，どんな方法で，どんな材料を

もってきて，どんな方法で作ったかで決まるのです。後で試験をしてもよくもならないし，悪くもなりません。

さらに，品質試験では必ずしもすべてのことを確認できません。医薬品の製造では，錠剤であれば何十万錠という大量のものを作りますが，試験はたかだか20錠くらいしかしません。ですから製剤設計および製造法設計が重要なのです。

【製剤設計の情報はどのように活用されるのでしょうか。】

審査側が設計の良し悪しの判断材料に情報を使う，企業側は情報を把握したうえで製造するという，両面があります。日本の医薬品承認申請資料のなかではとくに，製剤設計の情報が欧米に比べて非常に少ないといわれます。ですがICHのPDの議論では，必ずしも欧米だけが進んでいるというふうには思えません。国際的にどういう点が重要かを議論するには，1年，あるいは2年くらいかかかるかもしれませんが非常に意義が大きいことだと思います。

【PDのトピック化には日米欧のどこが積極的だったのですか。】

そうですね。欧米企業が積極的だったようです。日本企業の製剤設計に関する記述が少ないのと，いまはそうではないのですが，歴史的に開発初期からの製剤変更を薦めていなかったという背景があります。

製剤特性は製造スケールによって変わる可能性があり，とくに固形剤では製造の仕方，大きさを含めて性能，とくに体内での吸収が変わってきます。しかし日本ではかつて製剤変更に関し，製剤の組成を変えなければ製剤特性は同じだという考え方がありました。臨床試験の最初から最後まで同じ製剤を使っていたという過去のアプローチ，別の新たな知見が得られて変えた方がいいと思っても変えにくいという考え方を，欧米の企業は不調和だと考えているかもしれません。

しかし現在はこのような不調和は現実的にはないと思います。第1相から第2相へ移る際に製剤を変えてはいけないとは，日本の審査側は言っていません。ただ誤解はあるかもしれません。個人的には，昔のやり方でいいという考え方，要するにスケールが変わっても組成が変わらなければいいという考え方を持っている日本企業が少なからずあると思っています。いま話しました固形剤の製造スケールの違いは，今回のPDのガイドラインのなかで大きな項目のひとつになると思います。

【PDガイドラインが与える影響はいかがでしょう。】

企業には，製剤を製造していくなかで製造法，製造スケールを変えたときに製剤特性がどうかわるかということをきちんと把握しておく義務が生じます。審査では行政から，その点を質問されることになります。また，企業が工場での製造を続けていくうえでも製造法，スケールの変更の際には，適切な変更管理が必要です。企業が自主的にどの程度のデータを取らなければいけないかどうかはこれからの議論です。

一方，行政は，製剤設計・製造法設計の情報を審査段階でつかむだけでなく，市販後のいわゆるGMP監視の知識のベースにすることもPDガイドラインはめざそうとしています。監視機関が製造のどの部分がより重要であるかをつかみ，その部分を企業がきちんと管理しているかをみるわけです。審査では臨床開発段階での成功例をみているわけで，決して市販後のものをみているわけではありません。これからの審査では，市販後に企業がどのようにものを作っていくか，管理していくかをより注視すべきです。この承認前と市販後のつなぎは，日米欧ともあまり強くありません。

現在行われている品質の再評価の結果から，かつての製剤設計が悪かったのか，その後の変更管理が悪かったのかわかりませんが，先発品でも相当品質がばらついているものが少なからず見つかっています。

【GMPについても議論があったようですね。】

7月にベルギーであった専門家会議では，GMPが形骸化しているのではないかという指摘がありました。これまでのGMPを端的にいうと，「決められたことはそのままやる」という面があります。GMPの形骸化の一例をあげると，ある運転状況を管理するときに，「なぜそういった管理をするのか」と工場に聞いても，「ここは工場ですから研究開発でやっていることはわかりません」というような答えが返ってきがちです。開発から工場への情報が途切れ，やっていることの適格性情報がわからないまま行われているということです。本来なら，所定の運転状況ができなかったときにどんな影響がでてくるかわかっていないと管理はできないはずです。

形骸化についてもうひとついうと，いろんなGMPルールのなかで，メリハリがつかず，リスクマネジメントがうまく機能していないように思われます。ある想定される不具合，被害を想定しながら不具合がおきるシナリオを考え，その不具合を回避したり，和らげる方法を考慮し，またリスクを適切に知らせるというリスク管理のサイクルが，GMPでも医薬品の承認審査でもうまく機能していません。メリハリをつけるというのは，リスクの大きいところへ重点的に人的資源・時間・資金を使って手当てすべきということですが，どうもうまくいっていません。

アメリカFDAは昨年あたりから，リスクをベースにしたGMP，21世紀型のGMPについて国内で議論を始めていますが，世界に対してもリスク管理という考え方を入れてGMPを議論しようということを提案したのです。その結果，7月の専門家会議でリスク管理を品質関係の新トピックにするという提案がまとまり，本会議直前のステアリングコミッティーで採択されました。ICHのトピック番号は先のPDがQ8，リスクマネジメントがQ9です。

【リスクマネジメントについてもう少し説明をお願いします。】

ここでのリスク管理というのはGMPだけを対象にしたものではなく，医薬品の品質保証におけるリスク管理で，幅が広いものです。また，リスク管理はこれまでまったくなかったわけではなく，企業も行政も，ある程度自然にやってきています。しかし，それぞれが一貫しておらず，国際的にも不調和があったと思われます。

いままでのガイドラインは，企業が行政側に提出する資料を対象にしてきました。今回のリスク管理が従来のガイドラインと大きく異なるのは，対象が行政と企業と両方になることです。Q9では具体的にどうするかということは決まらないにしても，考え方を整理することになります。

行政も企業も人的資源は限られます。どこかを切り捨てるというのではなく，どこが大事なのかを見極めるという点で，PDとリスクマネジメントは密接なつながりがあります。Q9ではQ8から出される個別重要点に基づき，どういった分野にリスクマネジメントを使うべきかという原則を決めていくことになります。

例えばアメリカではGMP査察は法制化されていて，一事業所あたり2年に1回は査察に行くと義務づけられています。ただ実際には人員不足でできていません。職員数がおよそ1万人いるアメリカFDAでもそうです。どの国でも行政側は人員不足なので，重要性の高いところ，リスクの高いところをよく見るということになります。おしなべてルールのあるときに，リスクの高くないところを一生懸命みてもしょうがないという考え方です。

【リスクマネジメントの不具合で，どのようなデメリットがあるのですか。】

リスク管理がうまく機能しなかったことによる問題で，患者にとって一番よくないのは不良医薬品が市場に出回ってしまうことです。医薬品の回収が起こるのは，出荷時に問題がわからなかったということです。開発であれ製造管理であれ，なにか重要なリスクを見過ごしてしまったから起こったのです。

科学的に考えて問題があることが想定されれば，製造法，溶出規格が承認事項には入っていなくても

溶出試験などで自主的にリスク評価をやっておけば，先ほど述べた医薬品再評価でわかった状況にはならなかったはずです。承認規格があっていればいいんだという考え方が企業側，行政側にもあります。これは大いに反省すべき点です。行政が認可したからといって，科学的に未来永劫，品質保証されているわけではないのです。

また，錠剤の表面の黒点，これは賦形剤の焦げたものだったりするんですが，そういったものは患者へのリスクはほとんどありません。ところが日本では不幸にして，ビジネス上では回収という大きなリスクになり得ます。消費者からの不平が出てくることに関するリスクのみを考え，多大な労力をかけ検査するものの，溶出試験は古い承認規格にないからやらないというのでは，力の入れ方がどうもおかしいのではないでしょうか。

製造実績に基づき規格を立てる方法では，規格が規定値を外れても有効性，安全性に問題はないのに法的に出荷ができず，廃棄処分されたりする。そういったこともリスク管理の使い方のまずさだと企業側は指摘しています。

リスクマネジメントのガイドラインについてはまず，リスク管理の使い方に関する言葉の定義を重点的に，時間をかけて議論します。医薬品の品質に関してもリスク管理の考え方を作り上げます。ガイドラインは，来年の終わりごろまでに合意する予定です。

【そのほかの動きはどうですか。】

品質保証のシステムが話題になりました。具体的にいうと製剤のGMPガイドライン，Q7Aの次にくるガイドラインを作りたいということです。これは企業側からの要求がかなり強く，その意義はわたしも認めるところです。

既存のGMPガイドラインに，先ほどのPDとリスクマネジメントの部分を含めた総合的なものを作ることが提案されています。その考え方については，行政側はだれも反対しないのですが，品質関連ではふたつのトピックが新たなステージにあがったところなので，それらの方向性がみえてきてからの議論になるでしょう。今後の課題ですね。

【品質保証のシステムづくりの必要性について，どうお考えになりますか。】

GMPについて重要なのは，開発段階と市販後をつないだり，メリハリをつけたりするということです。どこが重要かを見極めるためにリスク管理の方法も確立する。もちろん，リスクを知りながらだまっているのは最悪ですから，リスク要因を解決しつつ中身についても情報提供することが重要になります。

これはアイデアの段階ですが，メリハリをつける意味で，例えば申請資料の製剤設計の部分に，「こういう設計をしました」とか，「それ以外のものではこういうリスクがあります」「こういうリスクは少ないです」というようなリスク分析の結果を書くことが考えられます。そうした記述を通じて，リスクに関する知識を企業から行政に出す，企業内でも研究開発部門から工場に出す，製造の委託元から委託先に出すということです。

リスク評価のなされた医薬品は，そうでない医薬品よりもトータルでのリスクは低いという考え方が出てきています。実際にアメリカの行政当局は，リスク管理のデータを提出した企業について，承認後の監視の程度を下げてもいいかもしれないとの見方を示しています。

【日米欧３極でのGMP標準化にも影響しそうですか。】

日米では，監視を行う職員数が違ううえ，行政がどこまで見なければならないかという考え方に相当大きな隔たりがあるように思います。アメリカのために日本の行政が委託を受けて行うとか，逆に日本のためにアメリカの行政が委託を受けるという相互認証は，個人的には実現性があるとは思えません。

まず考え方のベースをしっかり共通にしていくことが重要です。いまは同じ言葉を使ってもまったく

意味が違います。例えば企業間監査についていうと，日本ではGQPという話が出ていますが，元売の責任というのは当然のことです。自分に代わって業務を請け負ってもらっている会社がどんなプロセスで仕事をしているのか，ノウハウや品質には立ち入らない範囲でGMPでどの程度の管理をしているのかを知らなければ責任は取れません。

ですから原材料メーカーにも実際に行って監査することを，アメリカは期待するわけです。例えばアメリカの製薬会社が中国やインド，あるいは日本で原薬を作る場合，必ずその現場に国の査察官が行きます。当然ながら原薬を買う企業の品質保証担当者も出向きます。

一方，日本での企業間監査がどのように行われているかを調べてみると，わたしがみる限り現場に出向いたことがなく，書類審査だけという方法も存在するようです。そうなると，チェックリストを相手企業に送り，記入してもらってそれを見て判断するという監査と，海外にまで出かけていってその製造記録を見，担当者と会ってどうやっているかを聞いてチェックする監査とでは，両方とも「監査」と言ってもまったく程度が違う。企業間監査はそれくらい違うのです。これまで日本の化成品を対象とするGMP査察は，海外には出て行かず，どちらかといえば書類審査が主流なのです。効率を考えなくてはなりませんが，実際の監査の仕方に相当の開きがあるように思われます。品質関連の２つの新トピックが議論され，考え方のすりあわせができてくれば標準化は推進されるのでしょうが…。

【今後の課題はなんでしょうか。】

ICHガイドライン(CTD，PD)への対応に関し，企業研究者，薬事担当者，そして提出された申請資料を審査する行政，GMPの監視員の訓練が重要です。企業も行政もお互いに慣れていないので，そこが課題といえます。

Available online at www.sciencedirect.com
SCIENCE DIRECT
Biologicals 34 (2006) 65–68
BIOLOGICALS
www.elsevier.com/locate/biologicals

Regulatory perspectives from Japan – Comparability of biopharmaceuticals☆

Toru Kawanishi*

Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Accepted 22 August 2005

Abstract

In Japan there is no official guideline about comparability assessment of biotechnological products at present. However, there is some notifications which should be referred to, when the manufacturer changes the manufacturing process. Here, regulatory perspectives from Japan on the comparability assessment are presented. When establishing the comparability of biotechnological products derived from different manufacturing processes and the validity of modified manufacturing process, rational step-by-step approaches based on both product and process aspects would be useful. At first, relevant physicochemical and biological properties of products including purity, impurity profiles and stability should be compared before and after the manufacturing change, depending on the type and nature of the desired products. It is also necessary to examine the capacities of the new manufacturing process for ensuring the consistent production of the active protein product as well as the anticipated elimination of potential impurities and contaminants. Further relevant assessment of preclinical and clinical comparability of product may be necessary in some cases.

Keywords: Biotechnological product; Comparability; Japan; ICH-guideline; Regulatory perspectives; Harmonization

1. Introduction

Biotechnological products were developed and produced based on many innovative technologies, which are always advancing by themselves. The products are, therefore, often subject to change in the manufacturing process for improvement of the product quality and production economy, increase in production yield, and so on. It is not reasonable that the manufacturers are required to submit the same full data to obtain the authorization of the manufacturing change as to obtain the new drug authorization. USA-FDA and EU-CPMA have already set each guideline for comparability assessment of biotechnological/biological products. We had also started the discussion about the comparability guideline in Japan. However, we stopped developing it, because comparability assessment of biotechnological products was nominated as a candidate of the new topic in the ICH-Quality. Drafting of the harmonized guideline has just started in the ICH-EWG. Here, I would like to give the regulatory perspectives about the comparability assessment of biopharmaceuticals from Japan.

2. Present official notifications relating with comparability assessment of biotechnological products before and after manufacturing changes in Japan

In Japan, we do not have any official guideline for the comparability assessment of biotechnological/biological products whose manufacturing processes are changed, yet. However, there is a notification, which should be referred to, when the manufacturer changes the manufacturing process of biotechnology-derived drugs which have already been approved. That is the Notification No. 243 from the Pharmaceutical Affairs Bureau, MHW of 1984. However, nearly 20 years have already passed since the Notification was made and some parts

☆ The perspectives are before the discussion in the ICH-EWG.

* Tel./fax: +81 3 3700 9064.
E-mail address: kawanish@nihs.go.jp

1045-1056/06/$32.00
doi:10.1016/j.biologicals.2005.08.017

of the requirement are assumed to be too strict. At present we usually treat each case as summarized below.

The following recombinant drugs would be treated as "not new drugs", which are categorized as "1-(8) other drugs" in the Pharmaceutical Affairs Bureau Notification No. 698: the first is the product which contains identical active ingredient although the culture method is different from the approved drug; the second is that which contains identical active ingredient although the purification process is different from the approved drug; and the third is the other drug in which difference is not specified. The followings are also usually treated as "not new drugs" but decided on a case-by-case basis: the product which contains identical active ingredient but its structure gene is identified by different process; and the product which contains identical active ingredient but host cell/vector system is different from the approved drug. In the case of the category 1-(8) other drugs as "not new drug", the data on specification and test methods, stability, and bioequivalence are required to be submitted for the registration as the pharmaceuticals, and a list of literature references concerning toxicity, pharmacological action, absorption, distribution, metabolism and excretion, and clinical trials for active ingredients concerned, as well as an outline of the list contents and the results of evaluation test are also required. In addition, in the case of the biotechnology-derived drugs, the following data are also needed on a case-by-case basis:

data on the manufacturing process, physicochemical analysis, specifications and test methods, stability;
data on single dose administration toxicity in one species of animals;
data on bioequivalency study;
data on clinical study for safety, etc.

The present notifications relating with the comparability of the products between before and after the changes in the manufacturing process in Japan are very simple, as summarized above. However, we have discussed much how to assess comparability of biotechnological products to draft the guideline, within Japanese experts. The following is the perspectives obtained from the discussion.

3. Regulatory perspectives from Japan: "how should we assess comparability of biotechnological products before and after the manufacturing change?"

To date, various topics related to the characterization and quality assessment as well as the manufacturing process for biotechnological products have been the subject of ICH harmonized guidelines and have proven very useful, in allowing manufacturers to develop a global approach to these issues. However, there is no specific international guideline on comparability of biotechnological products subject to changes in the manufacturing process. The subject we are facing is how to develop and establish rational concepts and approaches for establishing comparability of protein products derived from different biopharmaceutical manufacturing processes.

3.1. When is comparability assessment needed?

A comparability assessment is needed when a manufacturer wants to claim that the product of new manufacturing process Y is comparable to the already existing product of manufacturing process X with respect to quality, safety and efficacy (Fig. 1). The new process Y would be employed by either the same manufacturer, innovator or by different subsequent-entry manufacturer(s). The existing product from process X may be either an already licensed one or one under development for new drug application for approval. In case where there is an already licensed drug, subsequent-entry product(s) from different manufacturer(s) will be dealt with as a so-called generic product(s). On the other hand, the application from the innovator will be handled as a partial variation from already licensed conditions for the drug with respect to the manufacturing process. In the case of manufacturing variation of the product under development, the issue becomes the verification of such change within a single manufacturer at various stages of product development from early stage research to pre-approval. Here, the followings should be mentioned: it has been already decided that the generic products are excluded from the scope of the ICH-Q5E comparability guideline, but in Japan we still think that the comparability of the generic products could be evaluated following the same scientific approach.

3.2. General principles of comparability assessment

When establishing the comparability of biotechnological products derived from different manufacturing processes and the validity of modified manufacturing process, rational step-by-step approaches based on both product and process aspects would be useful. In this approach, the following parameters should be considered as key points:

(1) physicochemical and biological characterizations;
(2) impurities profile and the presence of potential contaminants;
(3) batch analysis;
(4) product stability;
(5) manufacturing process evaluation/validation studies; and in wider perspective
(6) preclinical and clinical studies.

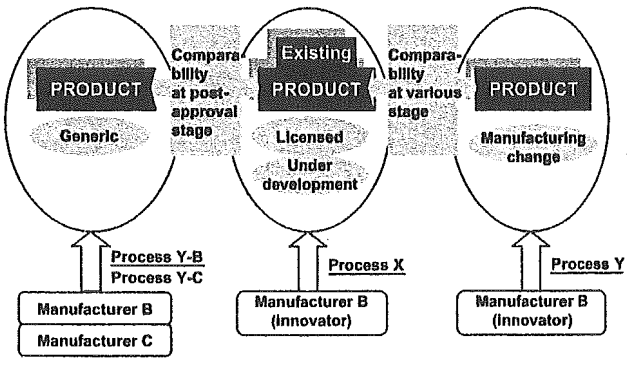

Fig. 1. Various cases of comparability assessment.

3.3. Strategies for comparability assessment

From the viewpoint of product aspects, the essential and critical first step is to establish whether the new candidate product in question is comparable to the existing product in terms of molecular and quality attributes. This is because whatever changes (minor or major) in the manufacturing process are made, if the new candidate product in question is not comparable to the existing product in terms of molecular and quality attributes, the new one will rather be regarded as a novel molecular entity for new drug application, but not as a qualified candidate for further comparability studies. The candidate product should be, therefore, the subject of extensive identification and characterization, as well as quality assessments including tests on impurities profile and the presence of potential contaminants. If these attributes of the candidate product and process are found to be comparable to those of the previous ones, further assessment of preclinical and clinical comparability would be performed, where necessary and appropriate.

3.4. Comparability from product aspects

Before going into some details about the need for further assessment of preclinical and clinical comparability, however, one should ask the following key question: "what is the identity or comparability of the biosynthetic protein product which possesses the inherent degree of structural heterogeneity?" In other words, what kind of criteria should be applied for establishing the identity or comparability of the candidate product(s) compared to the previous product with respect to molecular and quality attributes?

To answer this question, we should remind new concepts in the ICH-Q6B document. In the document we have introduced the concept, which has defined the desired product and variants, so that an inherent degree of structural and quality heterogeneity can be dealt within a relevant way. Desired product is defined as: (1) the protein which has the expected structure, or (2) the protein which is expected from the DNA sequence and anticipated post-translational modification (including glyco-forms), and from the intended downstream modification to produce an active biological molecule. When molecular variants of the desired product are formed during manufacture and/or storage and have properties comparable to the desired product, they are considered to be product-related substances and incorporated into active ingredient. When molecular variants of the desired product do not have properties comparable to those of the desired product, they are considered to be product-related impurities. In the concept, active ingredient may be composed of the desired product and multiple product-related substances; the desired product can be a mixture of several molecular entities derived from anticipated post-translational modification. Impurities may be either process-related or product-related (Fig. 2).

Various cases are considered for minimum qualification for further comparability assessments depending on each following specific type of desired product (A–D):

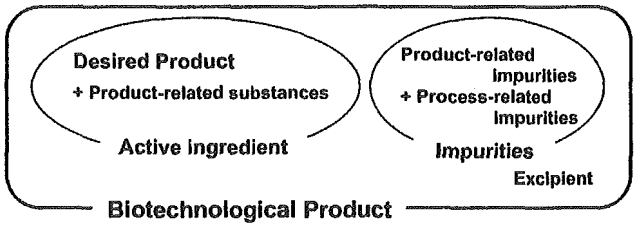

Fig. 2. New concept about biotechnological product in the ICH-Q6B.

(A) the protein which has the expected structure (e.g., monoclonal antibodies);
(B) the protein which is expected from the DNA sequence (simple protein);
(C) the protein which is expected from the DNA sequence and anticipated post-translational modification; and
(D) the protein which is expected from the intended downstream modification to produce an active biological molecule.

In the case of the "Desired product" being defined as the protein which has the expected structure, like monoclonal antibodies, minimum qualification for a candidate product for further comparability assessments should be that the product is derived from the same initial cell clone as a previous one and has comparable molecular and quality attributes compared to a previous one with respect to: (1) structural features, (2) physicochemical, (3) immunological properties, and (4) impurities profile. Variation of carbohydrate heterogeneity due to changes in culture conditions should be considered on a case-by-case basis.

In the case of the "Desired product" being defined as the protein which is expected from the DNA sequence, like recombinant insulin, minimum qualification for a candidate (product) for further comparability assessments should be that the product is the same as an already existing one with respect to protein structure, physicochemical and biological properties, as well as comparable impurities profiles.

In cases where the in vivo biological activity is closely related to the intended clinical effectiveness, further preclinical and clinical assessments with respect to efficacy may be omitted.

In the case of the "Desired product" being defined as the protein which is expected from the DNA sequence structure and anticipated post-translational modification, typically like glycoproteins, minimum qualification for a candidate product for further comparability assessments should be that the product is derived from the same initial cell clone as a previous product and has the same protein structure, comparable physicochemical properties, comparable carbohydrate patterns compared to a previous product with respect to the types of sialic acids and their contents, and antennary profile. Here, comparable biological properties, especially ensuring higher-order structure, in vivo activity and representing the clinical effectiveness, if any, is a critical factor for the qualification.

In the case of the protein which is expected from the intended downstream modification to produce an active

J Pharmacol Sci 97, 361 – 368 (2005)

Journal of Pharmacological Sciences

Full Paper

Simultaneous Real-Time Detection of Initiator- and Effector-Caspase Activation by Double Fluorescence Resonance Energy Transfer Analysis

Hiroshi Kawai[1], Takuo Suzuki[1], Tetsu Kobayashi[1], Haruna Sakurai[2], Hisayuki Ohata[2], Kazuo Honda[2], Kazutaka Momose[2], Iyuki Namekata[3], Hikaru Tanaka[3], Koki Shigenobu[3], Ryu Nakamura[4], Takao Hayakawa[1], and Toru Kawanishi[1,*]

[1]*Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[2]*Department of Pharmacology, School of Pharmaceutical Sciences, Showa University, Tokyo 142-8555, Japan*
[3]*Department of Pharmacology, Toho University School of Pharmaceutical Sciences, Chiba 274-8510, Japan*
[4]*Carl Zeiss Co., Ltd., Tokyo 160-0003, Japan*

Received August 31, 2004; Accepted January 8, 2005

Abstract. Fluorescence resonance energy transfer (FRET) with green fluorescent protein (GFP) variants has become widely used for biochemical research. In order to expand the choice of fluorescent range in FRET analysis, we designed various color versions of the FRET-based probes for caspase activity, in which the substrate sequence of the caspase was sandwiched by donor and acceptor fluorescent proteins, and studied the potential of these color versions as fluorescent indicators. Six color versions were constructed by a combination of cyan fluorescent protein (CFP), GFP, yellow fluorescent protein (YFP), and DsRed. Real-time monitoring in single cells revealed that all probes could detect caspase activation during tumor necrosis factor (TNF)-α-induced cell death as a fluorescent change. GFP-DsRed and YFP-DsRed were as sensitive as CFP-YFP, and CFP-DsRed also showed a large fluorescent change. By using two probes, CFP-DsRed and YFP-DsRed, we carried out simultaneous multi-FRET analysis and revealed that the initiator- and effector-caspases were activated almost simultaneously in TNF-α-induced cell death. These findings may give experimental bases for the development of novel techniques to analyze multi-events simultaneously in single cells by using FRET probes in combination.

Keywords: fluorescence resonance energy transfer, green fluorescent protein, tumor necrosis factor-α, cell death, caspase

Introduction

Many probes for various physiological reactions have been developed with green fluorescent protein (GFP) variants by using a similar strategy as that used with cameleon, the Ca^{2+}-sensing fusion protein developed by Miyawaki et al. (1 – 9). The cameleon consists of cyan fluorescent protein (CFP), calmodulin, M13 peptide, and yellow fluorescent protein (YFP). This fusion protein senses Ca^{2+} as the change of fluorescence resonance energy transfer (FRET) efficiency between CFP and YFP. Calmodulin binds M13 in the presence of Ca^{2+}, which causes conformational change in cameleon, resulting in a change in the distance between and relative orientation of CFP and YFP. This change alters the FRET efficiency from CFP to YFP; therefore, Ca^{2+} can be monitored as the fluorescent change (1).

CFP and YFP are the most frequently used pair for analysis by FRET. This pair is suitable for FRET analysis because the spectral overlap between the emission of the donor protein (CFP) and the excitation of the acceptor protein (YFP) is sufficient for energy transfer, and their ranges of fluorescence are far apart enough to be separated by measuring devices such as fluorescent microscopy (10). However, there are limitations for the CFP-YFP pair. It is impossible, for example, to use the CFP-YFP FRET probe for simultaneous measurement with other probes that are made of GFP variants or have

*Corresponding author. FAX: +81-3-3700-9064
E-mail: kawanish@nihs.go.jp.

fluorescein structure. If more choice of FRET probes is available from wider fluorescence ranges, it would allow us to analyze multi-events simultaneously occurring in living cells.

In this paper, we developed caspase-sensors of various colors by using cyan, green, yellow, and red fluorescent proteins and assessed their ability to detect the caspase activation in living single cells. Based on the findings obtained, we tried to perform multi-event FRET analysis and clarify the temporal relationships between biochemical reactions during cell death.

Materials and Methods

Plasmid construction

Plasmid encoding CY-sensor, YFP-peptide-CFP, was generated as previously reported (11). The sequence encoding 11 amino acids at the C-terminus of YFP was eliminated in this construct. The C-terminal truncated forms of the CFP (or GFP) gene were generated by PCR with primers containing the NheI site or BspEI site and pECFP-C1 (or pEGFP-C1; Clontech, Palo Alto, CA, USA) as a template, and the restricted fragment was inserted into the NheI/BspEI sites of the CY-sensor to generate a plasmid carrying truncated CFP (or GFP) at the N-terminus. DsRed was generated from pDsRed2-C1 (Clontech) by PCR, at the AgeI/NotI sites, and the restricted fragment was inserted into the AgeI/NotI sites of the CY-sensor to generate a plasmid carrying DsRed2 at the C-terminus. CG-, CR-, GR-, and YR-sensors were generated with a combination of these elements. The AgeI/BsrGI fragment from pEGFP-C1 was inserted into the AgeI/BsrGI sites of the CY-sensor to generate the GY-sensor. All cloned sequences were verified by sequencing.

Cell culture and transfection

HeLa cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 10% fetal calf serum (Invitrogen Corp., Carlsbad, CA, USA). Plasmid encoding the sensor protein was transfected into HeLa cells using Effectene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After 12 – 24 h incubation with the transfection reagent, the cells were washed with PBS and cultivated on dishes suitable for assay in medium containing 500 μg/ml of G418 for an additional 1 – 3 days until the assay was performed.

Western blotting

Cells cultured in a plastic dish were washed with PBS and lysed with 1 × SDS loading buffer. The samples dissolved in 1 × SDS loading buffer were incubated at 95°C for 2 min, and then they were loaded onto SDS-polyacrylamide gels (10%). Proteins were separated at 20 mA and then blotted to PVDF membranes in Tris-glycine transfer buffer at 100 V for 2 h. The membrane was incubated with block ace (Dainippon Pharmaceutical, Osaka) for 1 h, anti-GFP peptide antibody (Clontech, diluted with 0.1 × block ace to 1:1,000) for 2 h, and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Chemicon International Inc., Temecula, CA, USA; diluted with 0.1 × block ace to 1:10,000) for 1 h. The membrane was washed with TBS-T 3 times for 5 min after the incubation with the antibody. All of these incubations were performed at room temperature. The membrane was developed with the ECL chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

Measurement of fluorescent spectra of the sensors in HeLa cells

Spectral imaging was performed with LSM510META (Carl Zeiss, Jena, Germany) (12). Cells expressing one of the sensors were observed by excitation light at 458 nm (Ar laser), emitted fluorescence was separated by a grating, and the separated fluorescence were detected by 24 photomultiplier tubes (PMT) that were set to detect fluorescence at 468 – 714 nm. Each PMT detected fluorescence in the 10.7-nm wavelength range. So, the fluorescent spectrum at 468 – 714 nm was obtained with 10.7-nm resolution. Cell death was induced by incubation with tumor necrosis factor (TNF)-α (100 ng/ml) and cycloheximide (CHX, 10 μg/ml) for 6 h. Fluorescent spectra of living and dead cells were obtained from the whole cell region of normal-shaped and spherical cells, respectively.

Real-time imaging with FRET sensors

Transfected cells were cultured on a cover glass (25-mm diameter, 0.15 – 0.18-mm thickness) for 1 – 3 days. Cells were treated with TNF-α/CHX and then incubated under the usual culture condition for 1 – 2 h before analysis. Analyses were carried out by confocal laser-scanning fluorescent microscopy using a Carl Zeiss LSM510 system. During the observation, the media were buffered with 10 mM hepes buffer (pH 7.4), and the cells were maintained at 35 – 37°C. DIC images and grayscale images for fluorescence channels were obtained every 2 min unless otherwise described. Excitation lights for the FRET probe (458 nm for the CG-, CY-, GY-, and CR-sensors; 488 nm for the GR- and YR-sensors) were provided by an Ar laser with a 458 or 488 dichroic mirror. Images of the FRET probe were obtained separately for both donor and acceptor

Table 1. Measurement conditions for real-time analysis by LSM510

Sensor	Fusion protein[a]	Excitation (nm)[b]	Emission (nm)[c]		
			beam splitter	emission filter	
				donor	acceptor
CG	GFP-peptide-CFP	458	515	467.5 – 497.5	515 – 545
CY	YFP-peptide-CFP	458	515	467.5 – 497.5	515 – 545
GY	YFP-peptide-GFP	458	515	475 – 525	515 – 545
CR	CFP-peptide-DsRed	458	515	467.5 – 497.5	560 – 615
GR	GFP-peptide-DsRed	488	545	505 – 530	560 – 615
YR	YFP-peptide-DsRed	488	545	505 – 530	560 – 615

[a]N-terminal CFP, GFP, and YFP were in a truncated form in which 11 amino acids at the C-terminus were eliminated, and His_{10} was present at the C-terminus of CG, CY, and GY. [b]Excitation light was obtained by Ar laser and a 458 or 488 dichroic mirror. [c]Emitted fluorescence was separated by a 515 or 545 dichroic mirror, and the fluorescence of the donor and that of the acceptor were obtained through band pass emission filters.

fluorescence using a dichroic mirror and band-pass emission filters as shown in Table 1. Images were processed and quantified using MetaFluor software as follows: The average pixel intensity of the fluorescence of the whole cell region was determined for each channel. The ratio value was calculated as the average pixel value of the fluorescent ratio, (fluorescent intensity for the acceptor channel) / (fluorescent intensity for the donor channel), in the whole cell region. As cells changed their morphology during the observation, the whole cell region was determined separately in each image.

Results

Construction and characterization of FRET probes

We developed plasmids expressing caspase sensors as shown in Fig. 1a. A 12-amino-acid peptide derived from poly(ADP-ribose)polymerase (PARP) that is a well-known substrate of effector caspases was sandwiched by two different fluorescent proteins (an example of CFP-YFP is shown in Fig. 1a). The peptide sequence contains a caspase recognition site in the middle, and this fusion protein was cleaved mainly by caspase-3 (11). CFP-GFP, CFP-YFP, GFP-YFP, CFP-DsRed, GFP-DsRed, and YFP-DsRed were used as the donor-acceptor pairs. We named these fusion proteins CG-, CY-, GY-, CR-, GR-, and YR-sensor, respectively (Table 1). These fusion proteins show FRET in their intact form, whereas in the presence of active caspase, the peptide sequence is cleaved, CFP and YFP are far apart, and the fusion proteins do not show FRET any longer. The fluorescent ratio of acceptor/donor reflects the amount of FRET, so we used the reduction of this value as an index of caspase activation.

HeLa cells expressing one of these fusion proteins

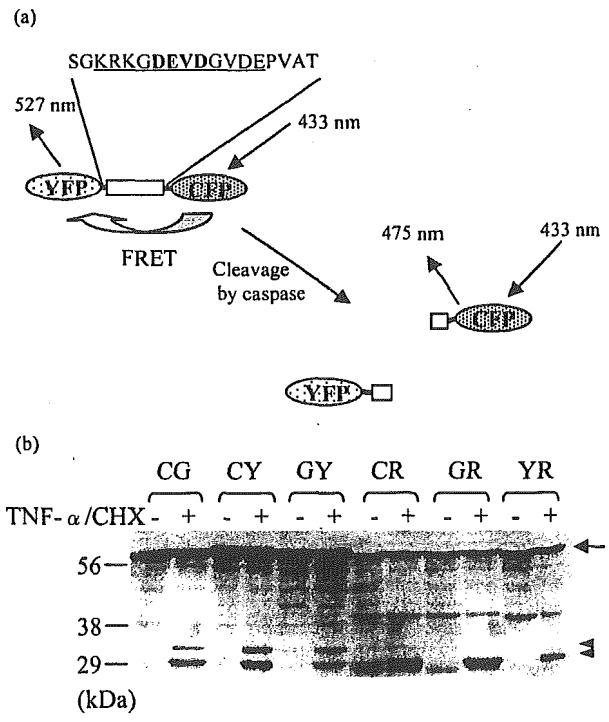

Fig. 1. Small peptide sandwiched by two different fluorescent proteins can be a caspase-sensor. a: Fusion protein that consists of a PARP-derived 12-amino-acid peptide sandwiched by CFP and YFP exhibits FRET in its intact form. In the presence of active caspases, the peptide is cleaved, and the fusion protein does not exhibit FRET. Caspase activation can be detected by measuring the fluorescence of CFP and YFP. b: Six caspase-sensors expressed in HeLa cells were cleaved by cell death stimuli. HeLa cells expressing one of the sensors were incubated in the presence or absence of TNF-α/CHX for 6 h. The arrow and arrowhead indicate the full length and cleaved fragments of the sensors.

were treated with TNF-α/CHX. After 6-h exposure, the sensor proteins in cells were extracted and analyzed by western blotting. All 6 fusion proteins were detected in their intact forms in non-treated HeLa cells (arrow in Fig. 1b), and small fragments were detected in cells treated with TNF-α/CHX (arrowhead in Fig. 1b), indicating that the fusion proteins were cleaved by cell death stimuli, as expected. The antibody used in this analysis reacts with CFP, GFP, and YFP, but not with DsRed. Therefore, CG-, CY-, and GY-sensor showed two cleaved fragments corresponding to the N- and C-terminal C/G/YFP, whereas CR-, GR-, and YR-sensor showed only one cleaved fragment corresponding to the N-terminal C/G/YFP.

Figure 2 shows the fluorescent spectra of the probes in living or dead cells. Comparing the fluorescence of living and dead cells, all sensors showed an increase of donor fluorescence and/or a reduction of acceptor fluorescence in response to cell death stimuli. This change results in a reduction of fluorescent ratio of acceptor/donor that is an index of FRET. These sensors were designed to show a reduction of FRET with caspase activation, so these results suggest that all 6 fusion proteins work as expected and can detect caspase activation as fluorescent change in living cells.

For simultaneous application of two or more fluorescent probes, minimum spectral overlap between probes is one of the important conditions. The spectra in Fig. 2 give us a clue to determine a suitable combination of probes for multi-probe analysis. CG-, CY-, or GY-sensor has the least fluorescence in the red-fluorescence region (>600 nm), so it is possible to use this fluorescent region for another dye. We can use a red-fluorescent dye that has fluorescence in this region together with CG-, CY-, or GY-sensor simultaneously. On the other hand, YR-sensor has the least fluorescence in the blue-cyan region (<500 nm), so blue-cyan-fluorescent dye is applicable with this probe for the purpose of simultaneous fluorescence imaging. The color variations of FRET probe may be useful for multi-probe analysis.

Real-time detection of caspase activation in living cells

Next, we applied the sensor proteins to real-time measurement. HeLa cells expressing one of the sensor proteins were analyzed with a time resolution of 2 min by laser-scanning confocal fluorescent microscopy. Figure 3 shows typical images (a) and fluorescent changes (b) during cell death. HeLa cells expressing GR-sensor were treated with TNF-α/CHX. An increase of donor protein fluorescence (GFP), a reduction of acceptor protein fluorescence (DsRed), and a reduction of the fluorescent ratio of acceptor/donor (DsRed/GFP) were observed in each cell at a different time. Caspases began to work at the point when the fluorescent ratio began to decrease.

All sensors showed similar changes, meaning that all sensors were useful for real-time detection of the caspase activation in a living cell, although the apparent sensitivity was different between sensors. In order to compare the sensitivity of these sensors to detect the caspase activation, the amount of the fluorescent change was calculated. We defined the start point and the end

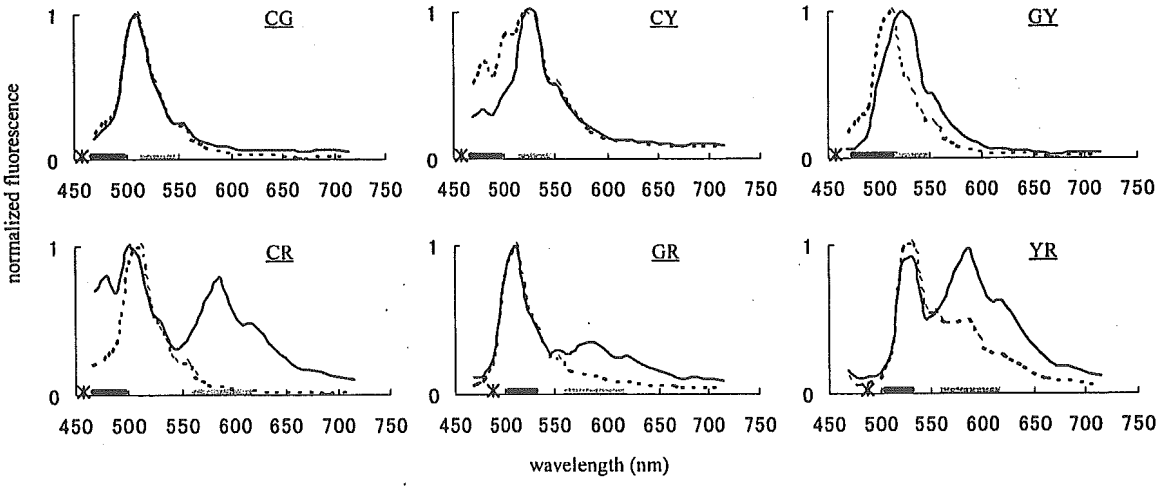

Fig. 2. Fluorescent spectra of the caspase-sensors in HeLa cells. HeLa cells expressing each sensor were treated with TNF-α/CHX for 6 h. The spectra of living cells (solid line) and dead cells (dotted line) were obtained from normal-shaped and spherical cells, respectively. Each spectrum was normalized to the peak that showed maximal intensity. The asterisks and bars on horizontal axes represent the excitation wavelength and detection range for the emitted fluorescence, respectively, used in real-time imaging analysis. Each spectrum is the average of data from 13 – 26 cells.

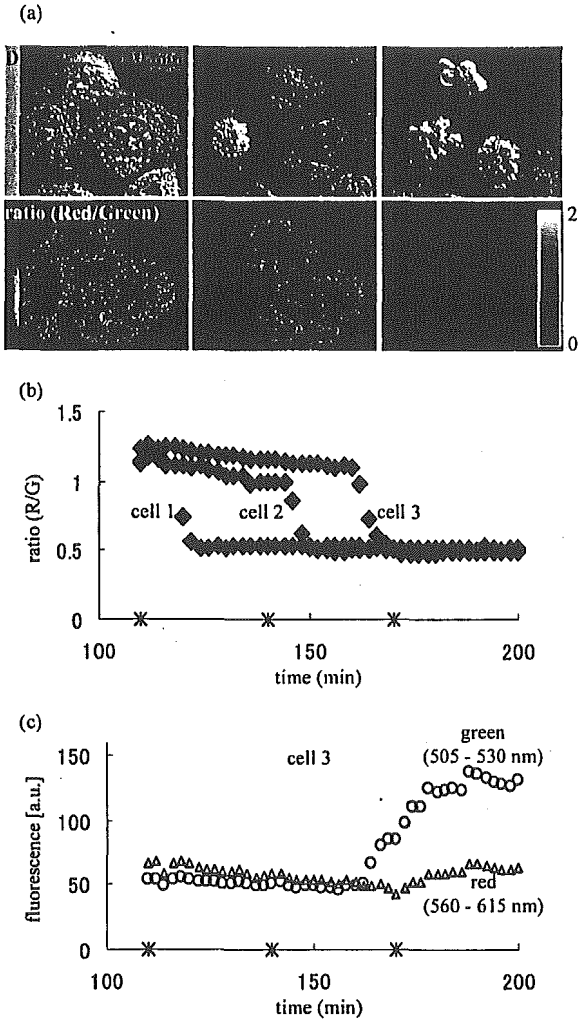

Fig. 3. Real-time imaging of caspase activation in living HeLa cells during cell death. HeLa cells expressing GR-sensor were treated with TNF-α/CHX, and fluorescent images were obtained every 2 min. a: DIC images (upper panels) and fluorescent ratios (Red/Green, lower panels) are shown in grayscale. The indicated time represents the time after the addition of TNF-α/CHX. Scale bar, 10 μm. b: The fluorescent ratio of cells were plotted. Cell 1, 2, or 3 corresponds to the cells shown in panel a. c: The mean pixel intensity in arbitrary fluorescent units (a.u.) for each channel was plotted. The fluorescence of cell No. 3 from panel a is shown. Open circle, GFP; open triangle, DsRed; closed diamond, ratio of Red/Green. Asterisks on the x-axis indicate the time points of the images in panel a.

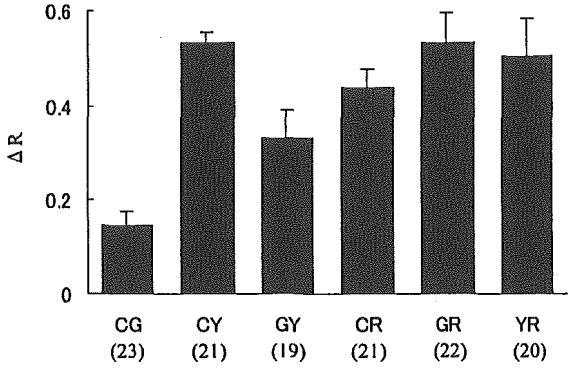

Fig. 4. Comparison of the sensitivity of various caspase-sensors. The amount of change of the fluorescent ratio during cell death (ΔR) was determined in each cell as described in the text. Bars represent means ± S.D. The number of cells used in each analysis is shown in parentheses.

point of the reduction of the fluorescent ratio as follows: the start point was the point after which the value decreased over four continuous points or more, the value decreased more than 10% in total, and the reduction of the value was not because of artificial noise such as focus shift; the end point followed the start point and was the point at which the value stopped decreasing. The sensitivity of the probe was calculated as $\Delta R = |(R_{end} - R_{start}) / R_{start}|$, where R_{start} and R_{end} were the fluorescent ratio at the start point and the end point, respectively. Figure 4 shows ΔR for each probe. GR and YR, as well as CY, showed the highest ΔR. They each showed a more than 50% change during cell death. CR showed a slightly lower ΔR, but its change was still 44% on average. CG and GY were less sensitive, probably because the fluorescent spectra of the donor and the acceptor were so similar that our system could not effectively measure FRET between them. CY *vs* GR, CY *vs* YR, or GR *vs* YR were not significantly different, and any other comparisons were significantly different by the Games-Howell test ($P<0.05$).

Simultaneous multi-event analysis using two FRET probes

Finally, we tried to perform multi-FRET measurement. We constructed a YR-initiator caspase sensor and a CR-effector caspase sensor by changing the caspase substrate sequence in the sensor and applied them to real-time imaging analysis simultaneously in order to reveal the temporal relationships between the initiator caspase activation and the effector caspase activation in the same cell. The caspase substrate sequences were derived from procaspase-3 and PARP, respectively, and their sequences are shown in Fig. 5a. These sensors were cleaved mainly by caspase-8/9 and caspase-3, respectively (11).

Simultaneous measurement of these sensors was performed under the multi-track scanning mode, in which two sets of excitation-detection conditions were used alternatively. CFP fluorescence by excitation at 458 nm was measured in the first track, and YFP and DsRed fluorescence by excitation at 488 nm was mea-

(a) YFP-SGLDCGIETDSGVDEPVAT-DsRed : YR-(IC)sensor
CFP-SGKRKGDEVDGVDEPVAT-DsRed : CR-(EC)sensor

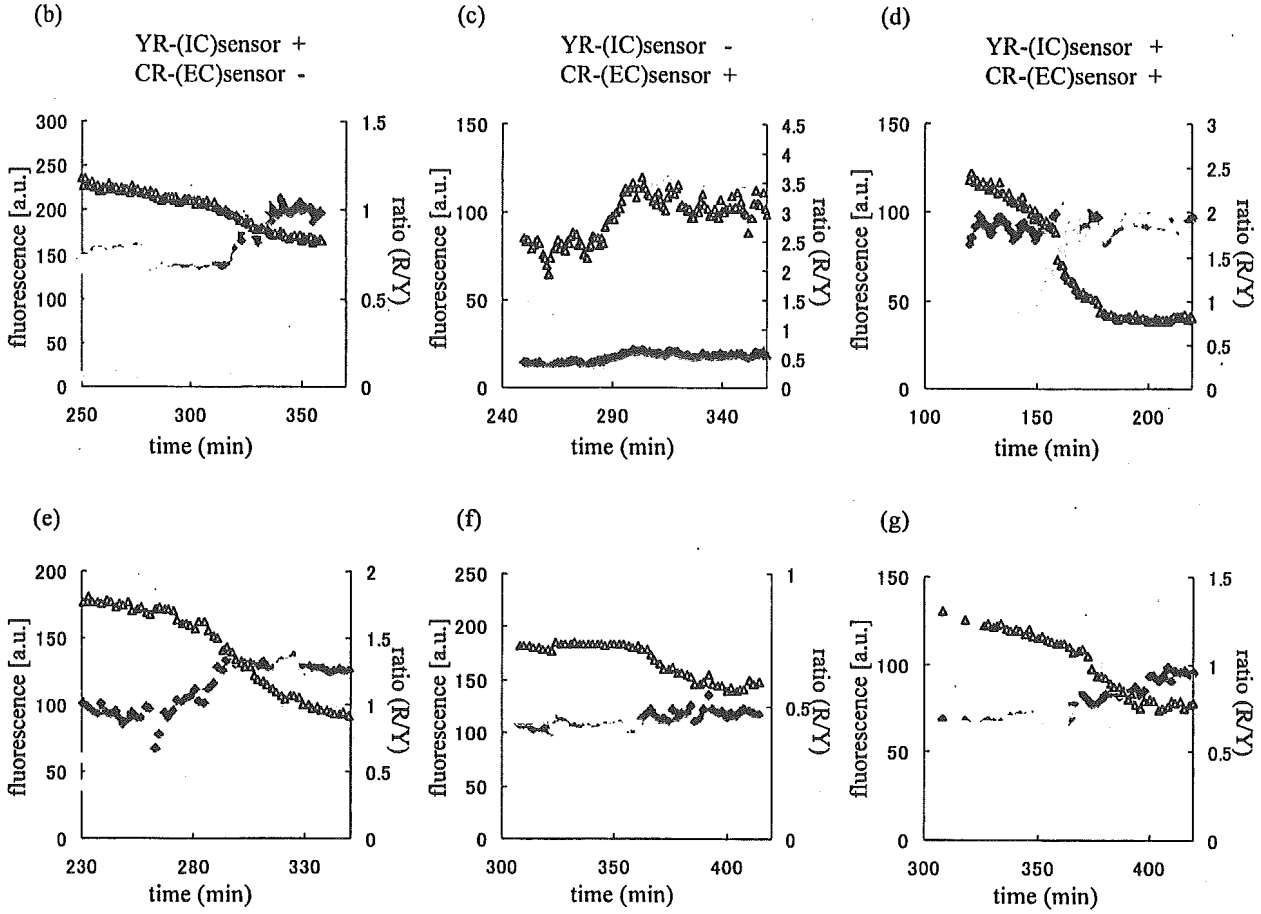

Fig. 5. Simultaneous measurement of initiator- and effector-caspase activation with YR-sensor and CR-sensor. HeLa cells expressing YR-initiator caspase sensor and/or CR-effector caspase sensor were treated with TNF-α/CHX and observed as described in the text. a: Probes used in this study. Underline indicates peptide derived from procaspase-3 and PARP, and bold indicates the consensus 4 amino acid sequence for caspase recognition. b – g: Cells expressing YR-initiator caspase sensor (b), CR-effector caspase sensor (c), or both of them (d – g) were treated with TNF-α/CHX. The fluorescence of CFP, YFP, and DsRed (colored plots) and the fluorescent ratio of DsRed/YFP (open triangles) were plotted against time after TNF-α/CHX treatment.

sured in the second track. The time difference of scanning between tracks is about 3 – 8 s. Figures 5b and 5c show control studies with cells expressing only one of the probes. These control studies were conducted in the same conditions as Fig. 5d. Figures 5b and 5c indicate that the YR- and CR-sensor could detect initiator- and effector-caspase activation as an increase of YFP and CFP signal, respectively, and the contamination of the signal between the YFP and CFP channels was negligible. So, we used an increase of the YFP and CFP signal as index of the initiator- and the effector-caspase activation, respectively. The DsRed signal in Fig. 5c was derived from direct excitation of DsRed in the CR-sensor by the excitation light at 488 nm and was increased when the cell shrank because fluorescent proteins were concentrated in the cell.

Figure 5d shows typical data of multi-probe analysis with the YR-initiator caspase sensor and CR-effector caspase sensor. In this cell, the fluorescence was dramatically changed at 150 – 160 min after TNF-α /CHX treatment. The YFP and CFP signal began to increase almost simultaneously, suggesting that initiator caspase and effector caspase were initially activated within a short time period. Figures 5e – 5g show three

other examples. We observed more than 30 cells in at least 3 independent experiments and found that all dying cells showed similar results.

Discussion

In this study, we developed various color versions of caspase-sensors with CFP, GFP, YFP, and DsRed and revealed that various combinations are applicable in FRET analysis. CY, CR, GR, and YR pairs are preferable FRET pairs that possess a high ability to detect the caspase activation.

The sensitivity shown in Fig. 4 represents the apparent FRET change that depends on the measuring system and was determined by three factors. 1) Intrinsic FRET efficiency: All 4 fluorescent proteins had different fluorescent characteristics; therefore, the levels of FRET efficiency in 6 probes differed from each other. 2) Excitation crosstalk: The acceptors were excited directly by the excitation light. 3) Emission crosstalk: The acceptor channel was contaminated with the donor signal, and vice versa, because the setting shown in Table 1 could not perfectly separate the signals from the donor and the acceptor. The differences in these factors cause the difference of sensitivity among the sensors. Factors 2) and 3) reduce the apparent FRET change in the measurement. In the case of the CG-sensor, for example, fluorescent spectrum of donor and acceptor are so similar that the intrinsic FRET efficiency may be high, but excitation and emission crosstalk may also be high, much higher than in other sensors (e.g., CY-sensor), resulting in the relatively low sensitivity of this probe in our measurement system. Crosstalk effects are undesirable for detection, but it is impossible to completely eliminate these effects in the current measurement system. Maybe we could obtain different results by using spectral imaging in which emission crosstalk is eliminated (12).

According to the characteristics of the fluorescence spectrum, the CY probe seems to be one of the best for FRET-detection. However, the probe is not suitable for imaging with confocal laser microscopy, because the normal argon ion laser, the most common one in confocal microscopes, is not suitable for the excitation of CFP. The blue laser is the most suitable for the excitation, but it is not common in confocal laser microscopes. In this paper, we had to use the argon ion laser emitting 458 nm and the special emission filters optimized for the confocal ratio-imagings of caspase activation using the CY probe (11). On the contrary, the GR probe and the YR probe can be efficiently excited at 488 nm emitted by the normal argon ion laser and imaged with a set of emission filters for fluorescein and a set for rhodamine, with which almost all of the confocal microscopes are equipped. In addition, the GR probe is useful for the detection of caspase activation in flow cytometry, because almost all of the normal flow cytometers are also usually equipped with the laser and the emission filters.

DsRed-containing "red"-sensors have several characteristics that are different from other "non red"-sensors. As previously reported (13), it takes longer for DsRed to mature and emit red fluorescence than it takes for GFPs, and DsRed fluorescence tends to decrease during real-time observation, which may cause a reduction of the apparent sensitivity. These characteristics must be considered when any analysis is performed with these sensors, but as shown in Figs. 4 and 5, red-sensors have a potential similar to that of the CY-sensor and are very useful for multi-color imaging.

It has been reported that DsRed is useful as a fusion tag and a partner for FRET (13, 14). Erickson et al. analyzed the potential of DsRed as a FRET partner with CFP and GFP (14). Mizuno et al. developed a Ca^{2+} sensing fusion protein using Sapphire and DsRed (13). And recently, Karasawa et al. used two novel fluorescent proteins, namely the cyan-emitted and orange-emitted fluorescent proteins from *Acropara* sp. and *Fungia concinna*, respectively, as a FRET pair, and measured caspase-3 activity in cells (15). These results combined with our results indicate that various fluorescent proteins including GFP derivatives, DsRed, and others are useful for FRET analysis. By choosing the appropriate two fluorescent proteins as the FRET pair, we can customize the fluorescent range of FRET-based imaging probes to fit the analysis, which would expand the flexibility of simultaneous multi-event analysis.

By using the CR and YR developed in this study, we were able to analyze two FRET probes simultaneously in the same cells. In several reports, the initiator caspase activity and the effector caspase activity were measured in living cells (8, 9, 11). In these reports, however, each activity was measured independently in different cells. To our knowledge, the present study is the first report that analyzes these activities in the same cell. The results directly reveal the temporal relationships between these caspase activities. It takes a long time for cells to start the initiator caspase activation after drug treatment, but it takes a relatively short time for cells to start the effector caspase activation after the initiator caspase activation. The caspase cascade is initiated at the last stage of cell death signaling, and it proceeds within a short time period.

Acknowledgments

This study was supported in part by a Grant-in-Aid for

Research on Health Sciences Focusing on Drug Innovation from the Japan Health Science Foundation; a Grant-in-Aid for Research on Advanced Medical Technology from the Ministry of Health, Labour, and Welfare; and a grant (MF-16) from the Organization for Pharmaceutical Safety and Research.

References

1 Miyawaki A, Llopis J, Heim R, Mccaffery JM, Adams JA, Ikura M, et al. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature. 1997;388:882–887.

2 Zaccolo M, Giorgi FD, Cho CY, Feng L, Knapp T, Negulescu PA, et al. A genetically encoded, fluorescent indicator for cyclic AMP in living cells. Nat Cell Biol. 2000;2:25–29.

3 Nagai Y, Miyazaki M, Aoki R, Zama T, Inouye S, Hirose K, et al. A fluorescent indicator for visualizing cAMP-induced phosphorylation in vivo. Nat Biotechnol. 2000;18:313–316.

4 Kurokawa K, Mochizuki N, Ohba Y, Mizuno H, Miyawaki A, Matsuda M. A pair of fluorescent resonance energy transfer-based probes for tyrosine phosphorylation of the CrkII adaptor protein in vivo. J Biol Chem. 2001;276:31305–31310.

5 Sato M, Ozawa T, Inukai K, Asano T, Umezawa Y. Fluorescent indicators for imaging protein phosphorylation in single living cells. Nat Biotechnol. 2002;20:287–294.

6 Tyas L, Brophy VA, Pope A, Rivett AJ, Tavare JM. Rapid caspase-3 activation during apoptosis revealed using fluorescence-resonance energy transfer. EMBO Rep. 2000;1:266–270.

7 Rehm M, Dussmann H, Janicke RU, Tavare JM, Kogel D, Prehn JHM. Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process: role of caspase-3. J Biol Chem. 2002;277:24506–24514.

8 Luo KQ, Yu VC, Pu Y, Chang DC. Measuring dynamics of caspase-8 activation in a single living HeLa cell during TNFα-induced apoptosis. Biochem Biophys Res Commun. 2003;304:217–222.

9 Takemoto K, Nagai T, Miyawaki A, Miura M. Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects. J Cell Biol. 2003;160:235–243.

10 Tsien RY. The green fluorescent protein. Annu Rev Biochem. 1998;67:509–544.

11 Kawai H, Suzuki T, Kobayashi T, Mizuguchi H, Hayakawa T, Kawanishi T. Simultaneous imaging of initiator/effector caspase activity and mitochondrial membrane potential during cell death in living HeLa cells. Biochim Biophys Acta. 2004;1693:101–110.

12 Zimmermann T, Rietdorf J, Pepperkok R. Spectral imaging and its applications in live cell microscopy. FEBS Lett. 2003;546:87–92.

13 Mizuno H, Sawano A, Eli P, Hama H, Miyawaki A. Red fluorescent protein from Discosoma as a fusion tag and a partner for fluorescence resonance energy transfer. Biochemistry. 2001;40:2502–2510.

14 Erickson MG, Moon DL, Yue DT. DsRed as a potential FRET partner with CFP and GFP. Biophys J. 2003;85:599–611.

15 Karasawa S, Araki T, Nagai T, Mizuno H, Miyawaki A. Cyan-emitting and orange-emitting fluorescent proteins as a donor /acceptor pair for fluorescence resonance energy transfer. Biochem J. 2004;381:307–312.

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2004; 18: 1156–1160
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/rcm.1465

Improved sensitivity for insulin in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry by premixing α-cyano-4-hydroxycinnamic acid matrix with transferrin

Tetsu Kobayashi*, Hiroshi Kawai, Takuo Suzuki, Toru Kawanishi and Takao Hayakawa
Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Received 27 February 2004; Revised 25 March 2004; Accepted 25 March 2004

This report describes an enhancement of the signal intensities of proteins and peptides in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). When α-cyano-4-hydroxycinnamic acid (CHCA) premixed with human transferrin (Tf) was used as a matrix, the signal intensity of insulin was amplified to more than ten times that of the respective control in CHCA without Tf. The detection limit of insulin was 0.39 fmol on-probe in the presence of Tf, while it was 6.3 fmol in the absence of Tf. The signal intensity of insulin was also enhanced when the CHCA matrix was premixed with proteins other than Tf (80 kDa), such as horse ferritin (20 kDa), bovine serum albumin (BSA, 66 kDa), or human immunoglobulin G (150 kDa). The optimum spectrum of insulin was obtained when the added amount of protein was in the range 0.26–0.62 pmol, regardless of the molecular weight of the added protein. Tf and BSA outperformed the other tested proteins, as determined by improvements in the resulting spectra. When the mass spectra of several peptides and proteins were recorded in the presence of Tf or BSA, the signal intensities of large peptides such as glucagon were enhanced, though those of smaller peptides were not enhanced. In addition, the signal enhancement achieved with Tf and BSA was more pronounced for the proteins, including cytochrome C, than for the large peptides. This enhancement effect could be applied to improve the sensitivity of MALDI-TOFMS to large peptides and proteins. Copyright © 2004 John Wiley & Sons, Ltd.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and electrospray ionization mass spectrometry have been widely used in studies of protein chemistry, including proteomics studies aimed at sequence identification or quantitative analyses following enzymatic digestion by isotope-coded affinity tags and other tagging systems.[1–8] In particular, MALDI-TOFMS has been used for the qualitative and quantitative analysis of intact proteins.[9–11] When the MALDI technique was first introduced as an ionization method for proteins, a mixture of fine metal powder and glycerol, or nicotinic acid, was used as the matrix.[12,13] Progress has been made with other matrix materials such as sinapinic acid, 2,5-dihydroxybenzoic acid (DHB), and α-cyano 4-hydroxycinnamic acid (CHCA), which have some desirable properties such as less intense adduct peaks and a relative insensitivity to contamination.[14–16] With the MALDI approach, analyte proteins are dispersed on a surface in a thin layer of matrix. The energy of an incident pulse of laser photons is absorbed by the matrix to form a jet of matrix vapor that lifts the analyte proteins from the surface and transforms some of them into ions.[13]

However, the mechanisms by which laser light irradiation is able to generate macromolecular ions have not been fully verified to date. It has been reported that the ionization of macromolecules by the MALDI process is affected by several factors. For example, peptide signal intensity was increased by the use of acetone as the solvent for CHCA matrix instead of employing the commonly used solvent, a mixture of acetonitrile and aqueous 0.1% trifluoroacetic acid (TFA).[17] The signal-to-noise (S/N) ratios for macromolecules are low in DHB matrix, but the addition of suitable additives (fructose, glucose, fucose, or 2-hydroxy-5-methoxybenzoic acid) to the DHB matrix improved its performance in the high molecular mass range.[18–21] In the CHCA and sinapinic acid matrices, the detection of higher molecular weight proteins was improved by using polytetrafluoroethylene (Teflon) as sample support.[22,23]

Recently, we investigated a method of identifying and quantifying proteins in blood using mass spectrometry. During the present study, we discovered that the signal intensity of human insulin was augmented more than 10-fold when transferrin (Tf) was mixed with the CHCA matrix

**Correspondence to*: T. Kobayashi, Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.
E-mail: kobayash@nihs.go.jp
Contract/grant sponsor: Ministry of Health, Labor and Welfare, Japan.

solution used for MALDI-TOFMS. This phenomenon was not specific to either insulin or Tf, which suggested that such enhancements could be used more generally to improve the sensitivity of protein analysis with MALDI-TOFMS.

EXPERIMENTAL

Materials

Human atrial natriuretic peptide (hANP), glucagon, insulin, insulin-like growth factor-1 (IGF-1), transferrin (Tf), bovine serum albumin (BSA), horse spleen ferritin (106 mg/mL in 0.15 M NaCl), and ProteoMass Peptide & Protein, were purchased from Sigma (St. Louis, MO, USA). Human immunoglobulin G (IgG, 11.3 mg/mL in 0.01 M sodium phosphate, 0.5 M NaCl, pH 7.6) was obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Human insulin, IGF-1, glucagon, and hANP stock solutions were prepared at concentrations of 100 pmol/μL by dissolving them in 0.1% TFA. Tf and BSA stock solutions were prepared at concentrations of 10 mg/mL by dissolving the materials in Millipore deionized water. ProteoMass Peptide & Protein stock solutions, which include bradykinin fragment 1-7, human angiotensin II, synthetic peptide $P_{14}R$, human ACTH fragment 18-39, bovine insulin oxidized B chain, bovine insulin, equine cytochrome C, equine apomyoglobin, rabbit aldolase, and BSA, were prepared at concentrations of 100 pmol/μL each, according to the manufacturer's instructions.

Sample application and data acquisition

The Tf-mixed CHCA was a 5:1 mixture of the CHCA solution (10 mg/mL in 50% acetonitrile in 0.1% aqueous TFA) and Tf solution (0.10 μg/μL; the final concentration was approximately 8.3 ng/μL), corresponding to 0.21 pmol Tf on each well of the target plate, if not otherwise noted. The control CHCA was a mixture of the CHCA solution and deionized water (5:1). A portion of each sample solution was immediately mixed with an equal volume of the matrix solution with or without Tf, and an aliquot of 2 μL (corresponding to 1 μL of sample solution) was applied to a stainless steel target plate. Mass spectrometric analyses were performed using an AB4700 proteomics analyzer (Applied Biosystems, Foster, CA, USA). The operating conditions were as follows: Nd:YAG laser (355 nm), linear mode, and detection of positive ions. The spectra were generated by signal averaging 50 laser shots into a single spectrum. The signal intensity was obtained after performing background correction and noise reduction using the Data Processor software (Applied Biosystems). This software was also used to determine the detection limit.

To confirm whether or not the matrix solution was at an optimum composition, serially diluted CHCA, DHB, or sinapinic acid solutions (from 10 to 0.078 mg/mL in 50% acetonitrile, 50% 0.1% TFA) were added to the insulin solution (100 fmol/μL). The most intense signal was obtained when 10 mg/mL CHCA was added to the insulin solution.

RESULTS AND DISCUSSION

Human insulin solution (6.3 fmol/μL) was mixed with an equal volume of Tf-mixed CHCA or control CHCA. When

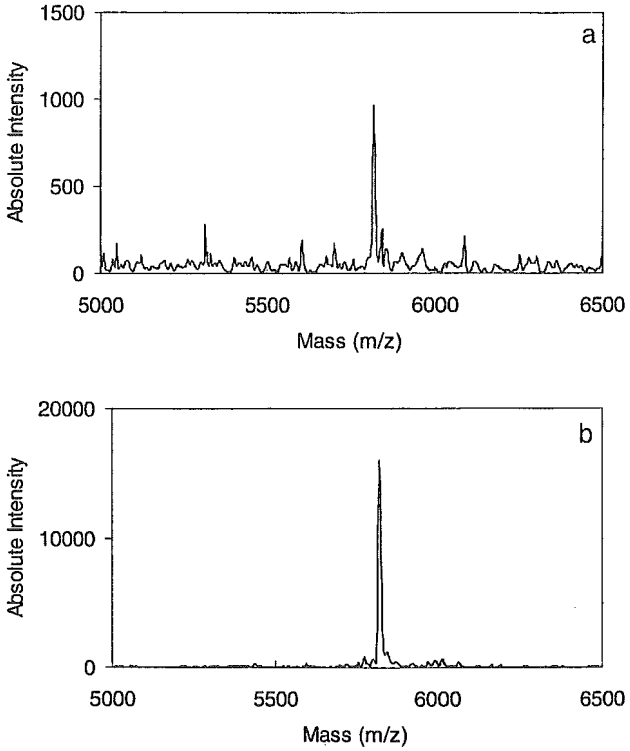

Figure 1. MALDI mass spectra of human insulin. The insulin solution (6.3 fmol/μL) and matrix solution were mixed together in equal volumes; 2 μL of the resulting mixture were applied to a target plate, allowed to dry, and analyzed by MALDI-TOFMS (see Experimental). The matrix solution was a 5:1 mixture of CHCA solution (10 mg/mL in 50% acetonitrile in 0.1% aqueous TFA) with deionized water or Tf solution (0.10 μg/μL). (a) Control CHCA used as matrix. (b) Tf-mixed CHCA used as matrix.

the Tf-mixed CHCA was used as matrix, the signal intensity of insulin in the MALDI-TOFMS detection system was amplified more than 10-fold relative to that achieved with the control CHCA (Fig. 1). To assess the sensitivity of insulin detection, the matrix solution was added to serially diluted insulin solutions (from 100 to 0.20 fmol/μL in deionized water), and samples were then spotted on a target plate. The detection limit of insulin was 0.39 fmol on the target plate in a Tf-mixed CHCA matrix under the present experimental conditions, whereas this limit was 6.3 fmol in the case of CHCA without Tf (Fig. 2).

To obtain the optimum concentration of Tf for the enhancement of insulin measurement sensitivity, the CHCA solution was mixed with serially diluted Tf solutions (from 1.0 μg/μL to 7.8 ng/μL) before addition to the insulin solution (100 fmol/μL). The signal intensity increased in a Tf-concentration-dependent manner (Fig. 3(a)). However, the S/N ratio decreased when the Tf concentration was more than 125 ng/μL (Fig. 3(b)), though it should be noted that the S/N value was still higher than the corresponding control value, i.e., 15 ± 7. A signal for 0.39 fmol/μL insulin was detected in the CHCA solution mixed with 0.1 μg/μL Tf (Fig. 2), whereas the signal for 1.6 fmol/μL insulin was not detected in the CHCA solution mixed with 1.0 μg/μL Tf (data not shown). These results suggest that the detection limit was also decreased in the presence of a high concentration of Tf.

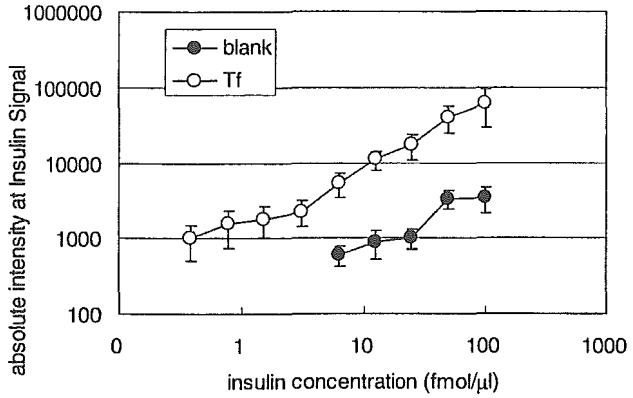

Figure 2. Dependence of insulin signals on insulin concentration. Sequentially diluted human insulin solution (100 to 0.20 fmol/μL in deionized water) and matrix solution were mixed in equal volumes. The matrix solution was a 5:1 mixture of the CHCA solution with either deionized water or Tf solution (0.10 μg/μL). The absolute intensity of the insulin signal obtained from Tf-mixed CHCA (open circles) is compared with that obtained for the control CHCA (closed circles). Each point represents the mean ± S.E. of four tests.

It is known that an excess amount of protein components can strongly influence the behavior of the MALDI process, resulting in partial or complete ion signal suppression.[24] In addition, the optimum mass ratio between the analyte and matrix for MALDI analysis has been demonstrated empirically.[15] When the CHCA was mixed with 1.0 μg/μL Tf, the excess amount of Tf might have suppressed the signal intensity of insulin as well. However, if that amount is appropriate, Tf appears somehow capable of enhancing the signal.

To determine whether or not the enhancement of the insulin MALDI-TOFMS signal intensity was specific to Tf, the CHCA solution was mixed with serially diluted solutions of several peptides and proteins before its addition to the insulin solution. The insulin signal intensity was also enhanced in the presence of ferritin (20 kDa), BSA (66 kDa), or IgG (150 kDa) (Fig. 4(a)). However, this was not found to occur in a simple concentration-dependent manner in the case of either ferritin or IgG; furthermore, when the CHCA solution was mixed with more than 2.0 μg/μL of these protein solutions, no insulin signal was detected. The enhancement of the insulin signal intensity was relatively small in the presence of peptides such as hANP (3.1 kDa) and glucagon (3.4 kDa). In addition, when the CHCA solution was mixed with more than 77 ng/μL of hANP or 87 ng/μL of glucagon, no insulin signal was detected. Among the tested peptides and proteins, the insulin signal intensity was enhanced most effectively in the presence of Tf (80 kDa) or BSA. Therefore, it is probable that this type of enhancement requires an added protein of moderate molecular weight, namely 66–80 kDa.

With regard to the results for the serial dilutions of the added peptides and proteins, the highest S/N values were obtained at 4.8 ng/μL hANP, 5.4 ng/μL glucagon, 66 ng/μL ferritin, 0.13 μg/μL BSA, 0.13 μg/μL Tf, or 0.57 μg/μL

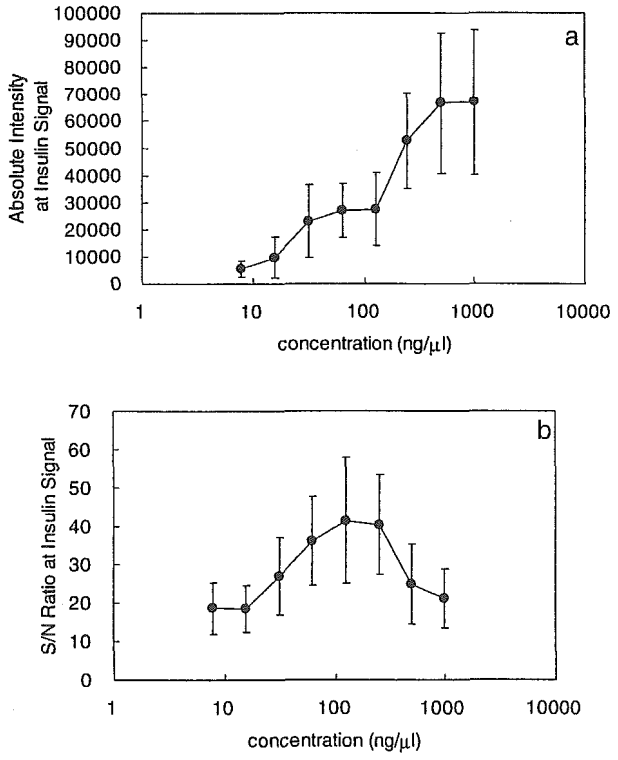

Figure 3. Dependence of insulin signal on Tf concentration. Serially diluted Tf solution was added to five volumes of the CHCA solution before mixing the resulting solution with an equal volume of human insulin (100 fmol/μL): (a) absolute intensity (arbitrary units) and (b) S/N ratio of the insulin signal in the MALDI analysis. Each point represents the mean ± S.E. of four tests.

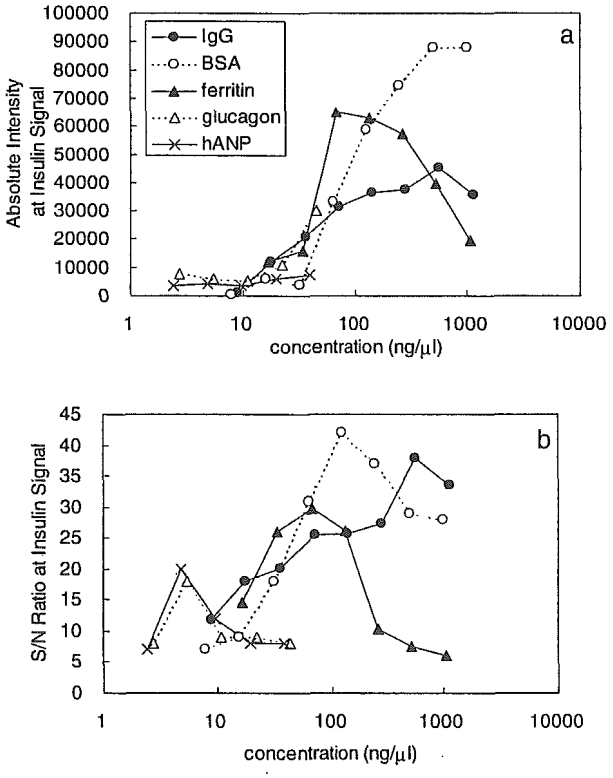

Figure 4. Dependence of insulin signal on concentrations of various added proteins. Serially diluted IgG, BSA, ferritin, glucagon, or hANP solution was added to the CHCA solution before the solution was mixed with the human insulin solution (100 fmol/μL): (a) absolute intensity (units) and (b) S/N ratio of the insulin signal. Each point represents the average of duplicate samples.

IgG (Figs. 3(b) and 4(b)), which correspond to 0.26 pmol, 0.26 pmol, 0.50 pmol, 0.32 pmol, 0.26 pmol, and 0.62 pmol, respectively, in each well. Thus, the optimum molar concentrations occurred in the same scale order, although the optimum mass concentrations of polypeptides required to enhance the signal differed markedly between the proteins and small peptides. In addition, the molar concentrations of excess peptides or proteins required to suppress the insulin signal were also found to exhibit the same scale in the same order. The ionization of insulin appeared to depend on the molar concentration of the peptide or protein which was mixed with the CHCA matrix solution.

To examine whether or not the signal enhancement was specific to human insulin, the CHCA solution premixed with Tf or BSA (0.10 μg/μL) was added to a solution of peptides and proteins, which included hANP, glucagon, human insulin, IGF-I, and ProteoMass Peptide & Protein at concentrations of 50 fmol/μL each. The signal intensities of [angiotensin II]$^+$ (1046 Da), [synthetic peptide $P_{14}R$]$^+$ (1534 Da), and [ACTH fragment]$^+$ (2465 Da) were either not enhanced or were reduced in the matrix premixed with Tf or BSA (Table 1). However, the signal intensities of [hANP]$^+$ (3080 Da), [glucagon]$^+$ (3483 Da), [insulin B chain]$^+$ (3494 Da), and [bovine insulin]$^+$ (5730 Da) were enhanced as well as that of [human insulin]$^+$ (5808 Da) (Table 1, Fig. 5). The signal intensities of [IGF-I]$^+$ (7649 Da), [cytochrome C]$^+$ (12 362 Da), and [cytochrome C]$^{2+}$ were enhanced more than that of human insulin in the presence of Tf or BSA. In addition, the signals of [apomyoglobin]$^+$ (16 952 Da) and [apomyoglobin]$^{2+}$ were clearly observed in the presence of Tf or BSA, although their signals were not detected in the control matrix. In this latter case, the signal of [apomyoglobin]$^+$ overlapped with that of BSA, but not of Tf; therefore, it was more advantageous to use Tf than BSA for detecting this signal. Since BSA was included in the ProteoMass Peptide & Protein solution, the signals of [BSA]$^+$ (66 430 Da), [BSA]$^{2+}$, [BSA]$^{3+}$, and [BSA]$^{4+}$ were also detected in the presence of Tf (Table 1, Fig. 5(b)).

The results reported above demonstrate that the enhancement of the signal intensity achieved with the use of Tf and BSA was observed for both peptides and proteins, and this effect was not specific to human insulin. The degree of enhancement was dependent on the molecular weights of the peptides and proteins, and no such enhancement was observed in the case of small peptides; in this regard a dividing line appeared to exist between [ACTH fragment]$^+$ (2465 Da) and [hANP]$^+$ (3080 Da).

The mechanism by which signal intensity enhancement was achieved with the use of peptides and proteins mixed with the matrix solution remains unclear. However, when super DHB (a co-matrix of DHB and 2-hydroxy-5-methoxybenzoic acid) was used as the matrix, ion yields and S/N ratio improved, especially for the high-mass range.[20] It has been suggested that this signal enhancement was caused by a disorder in the DHB lattice, allowing 'softer' desorption. This type of signal enhancement has also been observed in the case of substance P in CHCA after fast evaporation of an acetone solvent, which resulted in the more homogeneous distribution of matrix and analytes.[18] In addition, better mass resolution has been observed in the spectra of cytochrome C in a CHCA matrix desorbed from polyethylene and polypropylene membranes than has been observed with a CHCA matrix desorbed from stainless steel; it was thus suggested that such improved resolution might be due at least in part to the formation of relatively small matrix crystals within the membrane lattice structure.[25] In the present study, Tf and other proteins might have led to a similar disorganization in the CHCA lattice, resulting in the homogeneous distribution of insulin in the CHCA. However, the mechanism may differ from that suggested here, since the disorder in the CHCA lattice cannot reasonably account for why both Tf and BSA were able to enhance the insulin signal more effectively than either hANP or glucagon. As the next step, we are now planning to compare the crystals of the additive macromolecules plus matrix with those of the control matrix, using microscopic examination, to help elucidate the enhancement mechanism. We also intend to investigate whether the enhancement effect is observed in matrices other than CHCA. If crystallization is important,

Table 1. Signal intensities for proteins and peptides obtained using a matrix premixed with deionized water or with solutions of Tf or BSA

	Water	Tf	BSA
[Angiotensin II]$^+$	27 834 ± 10 757	17 057 ± 5021	19 755 ± 11 237
[P14R]$^+$	41 689 ± 15 289	30 675 ± 8588	29 237 ± 13 330
[ACTH 18–39]$^+$	4371 ± 1586	3801 ± 2246	5458 ± 3826
[hNAP]$^+$	5158 ± 1323	6889 ± 2879	9523 ± 6384
[human glucagon]$^+$	435 ± 183	674 ± 324	978 ± 566
[insulin B chain]$^+$	367 ± 257	997 ± 251	715 ± 479
[bovine insulin]$^+$	639 ± 100	6266 ± 2736	7498 ± 5331
[human insulin]$^+$	1267 ± 130	13 321 ± 5057	12 982 ± 6863
[equine cytochrome C]$^{2+}$	166 ± 83	5668 ± 1975	3460 ± 1442
[human IGF-I]$^+$	459 ± 81	7667 ± 1808	6263 ± 2872
[equine apomyoglobin]$^{2+}$	nd	2249 ± 994	2217 ± 1087
[equine cytochrome C]$^+$	114 ± 43	7629 ± 1804	4006 ± 1981
[BSA]$^{4+}$	nd	52 ± 14	2459 ± 604
[equine apomyoglobin]$^+$	nd	1347 ± 700	2090 ± 1316
[BSA]$^{3+}$	nd	155 ± 13	3721 ± 1426
[BSA]$^{2+}$	nd	114 ± 27	3624 ± 1681
[BSA]$^+$	nd	25 ± 8	634 ± 433

Each entry is the average of the most intense signals from four samples. nd: no signal was detected.