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CANCER ENZYMOLOGY

Edited by
J. SCHULTZ
F. AHMAD

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PAPANICOLAOU CANCER RESEARCH INSTITUTE,

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PREFACE

In recent years a great deal of progress has been made on the covalent modifications of proteins and how they affect physiological function. One of these modifications that profoundly affects many cellular functions involves proteolysis. Therefore, the main theme of the Eighth Miami Winter Symposia arranged by the Department of Biological Chemistry, University of Miami, concerns "Proteolysis and Physiological Regulation." As in the past, the program arranged by the Papanicolaou Cancer Research Institute is complimentary to this basic theme. This year we chose "Cancer Enzymology."

The selection of Dr. Hugo Theorell as the Lynen Lecturer made it possible for us also to select old friends and students of Dr. Theorell: namely, John Buchanan and Britton Chance. It also brought back to the symposia previous participants such as Sidney Weinhouse, Ronald Estabrook, George Weber, and Seymour Klebanoff. The plan of the program started with the strategy of the cell and progressed to bioenergetics, regulation and cytotoxicity, thus providing a role for enzymes in the mechanism of the living as well as protection against adverse environment.

It has always been a pleasure to meet with those who have attended the symposia in the past and continue to do so, each time exercising and expressing their pleasure at the format and general atmosphere of this Gordon Conference-like meeting taking place in mid-winter. The restful atmosphere in Miami at this time of year is conducive to more relaxed discussion and interaction among the participants. Such interaction is not readily possible at large national meetings where both the subject matter and the number of participants limits the opportunity to meet informally and enjoy the criticism of objective observers. It is, therefore, our sincere hope that future Miami Winter Symposia will be attended by many former participants, as well as many new scientists, to share in a mutually stimulating week of academic exchange.

We would like to acknowledge the support of Eli Lilly and Company, Abbott Laboratories, Hoffman-LaRoche, Inc., MC/B Manufacturing Chemists, and the Upjohn Company. Finally, it is of particular importance that the recording of the discussions and the preparation of this volume was made possible through the combined efforts of our expert typists, Kathi Bishop, Bonnie Tracy, and Anne Johnson, who worked under the direction of Mrs. Ginny Salisbury.

J. Schultz
F. Ahmad

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PROTEASE-RELATED EFFECTS IN NORMAL AND TRANSFORMED CELLS

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INTRODUCTION

The basic problem of cell biology concerned with the mechanism of stimulation of resting or dormant cells to proliferation has been approached in several ways. There is now a relatively long list of compounds that have been shown to be mitogenic for certain cell systems. This list includes serum (1-3), the protein hormones (4), insulin and insulin-like materials, such as multiplication stimulating activity (5) and somatomedin (6), fibroblast growth factor (7) and epidermal growth factor (8). Other biological materials including fetuin (9), seromucoid (10), lipopolysaccharide (11), glucocorticoids (12) and lectins (11) have also been reported to be mitogenic.

A third group of mitogenic substances, the proteases, have been of particular interest to us since the observation by Sefton and Rubin (13) that trypsin stimulates resting chick embryo cells to division and the report by Burger (14) in the same year that trypsin, ficin, and pronase can stimulate the growth of density-inhibited 3T3 cells. At the time of initiation of studies in our laboratory the role of proteases as mitogens had not been generally explored in many cell systems.

The use of proteases as mitogens seemed particularly attractive initially because they had been reported to react with proteins on the cell surface causing specific alterations that have been proposed to have special significance in the basic mechanism of the processes leading to cell division (15,16). Specifically, a cell surface protein of 250,000 daltons that can be enzymatically iodinated is removed by trypsin (17-20) and is absent from the external surface of many transformed cells (17-25). Furthermore, a protease capable of activating plasminogen has been indirectly implicated in maintaining the phenotype

of transformed cells (26-30). Thus, proteases have been proposed as active agents in the metabolism of both normal and transformed cells.

At the onset of our experiments we felt that certain restrictions should be applied to the choice of a protease selected for study.

It should

1) be a major constituent of the circulating fluids normally found in blood, and therefore in plasma, as a proenzyme, which can be activated if the need for cell proliferation occurs,

2) be readily available in relatively large quantities in highly purified form,

3) exhibit a high degree of proteolytic specificity,

4) serve as a mitogen for a host cell that will respond to this enzyme when present alone without the requirement of any other survival, attachment or growth factors.

Of the several possible choices among the kallikreins and the proteases of the clotting and complement systems, thrombin was selected as most nearly fulfilling these qualifications. Its proenzyme form, prothrombin, is present in blood at a level of 100 γ per ml. Serum contains active thrombin since the latter is produced in excess of the capacity of antithrombin proteins to neutralize it (31). The relationship of thrombin to other blood proteases is shown in Fig. 1.

Thrombin is a highly specific proteolytic enzyme that is known to split, for example, 4 arginyl-glycine bonds of fibrinogen (32) and a few selected lysyl and arginyl bonds of actin (33). It has been supplied to us in highly pure form from bovine and human sources by Dr. David F. Waugh and Dr. John W. Fenton, respectively.

RESULTS

Thrombin as a mitogen:

When added as a single component to the culture medium

of chick embryo fibroblasts, thrombin is capable of stimulating these cells to division (34). The assay for mitogenicity consists of measuring the response of resting cells for DNA synthesis from labeled thymidine 12 hr after addition of the reagent or after 4 days for total cell count.

By both criteria thrombin was highly mitogenic. When compared to graded levels of serum ranging from 0 to 5 percent, thrombin was equally effective when added between 0 and 10 μg per ml. Prothrombin, itself, was inactive but when incubated in a Ca^{++}-containing medium to which Factor X_a and V had been added in very small quantities, the proenzyme was converted to the active agent, thrombin. In this conversion the chick cells themselves provided the thromboplastin, a colloidal lipoprotein necessary for this reaction (see Fig. 1).

We then explored the relative proportions of thrombin-yielding components of defibrinogenated plasma to other mitogenic activities as calibrated by use of the chick embryo fibroblast as the host cell. The answer to this question was first approached by determination of the inhibition of the mitogenic activity of thromboplastin-treated plasma with phenylmethylsulfonylfluoride, which combines with and inactivates the "serine" proteases (32). Approximately 50% of the mitogenic activity of plasma was lost by this procedure. However, treated-plasma may contain other "serine" proteases with mitogenic activity, but in far less quantities than thrombin. One example is Factor X_a, which has been shown in this laboratory to be mitogenic (unpublished results).

Therefore, in order to assess the true contribution of prothrombin in plasma we separated the proteins in defibrinogenated plasma that are absorbed to $BaSO_4$ and tested the mitogenic activities of both the non-absorbed (Fraction I) and absorbed proteins (Fraction II) before and after treatment with thromboplastin. It was found that the total mitogenic activity of Fraction I did not depend on treatment of the preparation with thromboplastin, but that the activity of Fraction II was greatly enhanced by this treatment. When chromatography was performed on either the untreated or treated samples of Fraction II, we could show that in the former case the mitogenic activity was eluted with prothrombin and in the latter instance with thrombin. The mitogenic activity from thrombin precursors of plasma

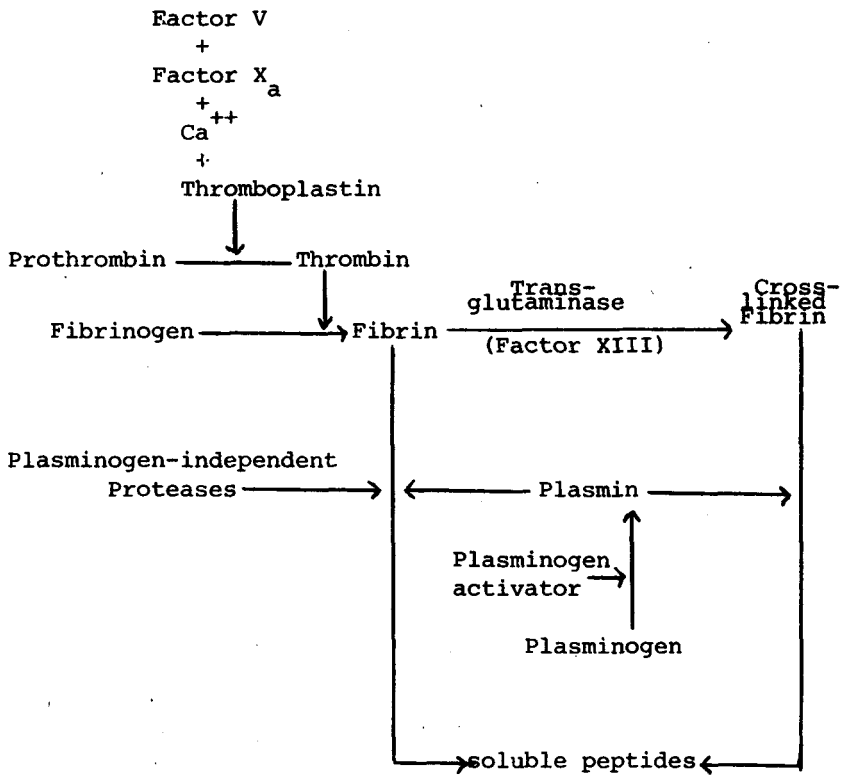

Fig. 1. Enzyme systems involved in the formation and dissolution of fibrin clots.

is approximately 30 percent of the total mitogenic activity. Thus, by either method of analysis, i.e. by specific analysis or by enzymatic inhibition with phenylmethyl-sulfonylfluoride, the thrombin-yielding material of plasma represents an important share of the mitogenic activity of this fluid. Therefore, from a quantitative point of view thrombin formed from prothrombin might be expected to play a role under certain physiological conditions, for example in wound healing. The concentration of thrombin in the microenvironment of the wound during clot formation probably

is considerably greater than that of collected serum and would be sufficient to initiate cell proliferation in the wound area, a necessary second step in restoring the damaged area to normal condition.

Since these experiments were conducted entirely with secondary cultures of chick embryo fibroblasts, we then examined the role of thrombin in other cell systems. When added to several murine embryo fibroblasts (mouse-Swiss, mouse BalB C, Rat, and Golden Hamster) thrombin was also strongly mitogenic. In a very surprising manner it could not under these conditions cause cell proliferation when tested on chick embryo fibroblasts transformed with Rous sarcoma virus. Whether this lack of response to thrombin represents a requirement for other serum factors or whether the transformed cell simply could not respond to the protease as a growth stimulator required further investigation.

There are certain cell lines that are unresponsive to thrombin when the latter is present as the sole mitogen, for example, 3T3, 3T6, CHO, BHK and Nil. In recent experiments we have examined the effects of thrombin on some of these cells when it is added in combination with other mitogenic factors.

In Fig. 2 we show results of an experiment with a mouse BalB C, 3T3 fibroblast cell line infected with Avian sarcoma virus B77. As the accessory factors we selected Cohen's epidermal growth factor (8) and prostaglandin F2α. The latter has been demonstrated to have a stimulatory effect on DNA synthesis and cell proliferation of Swiss 3T3 cells (35). The B77-3T3 line cell was used in these experiments because we have found that it is particularly responsive to epidermal growth factor. The cell count in all cases was normalized to the growth response obtained with 5% calf serum.

At a low concentration of serum (0.7%) B77-3T3 is maintained in culture but exhibits only minimal growth. When provided with thrombin at a level of 0.2 μg/ml there was no growth beyond this basic level. Highly purified epidermal growth factor supplied at a level of 1.5 ng/ml evinces a response 1.4 times that of the 5% serum level. At a level of 0.6 ng/ml the response is only 50%. It is at this latter concentration that effects of other factors can be demonstrated. For example, addition of prostaglandin F2α at

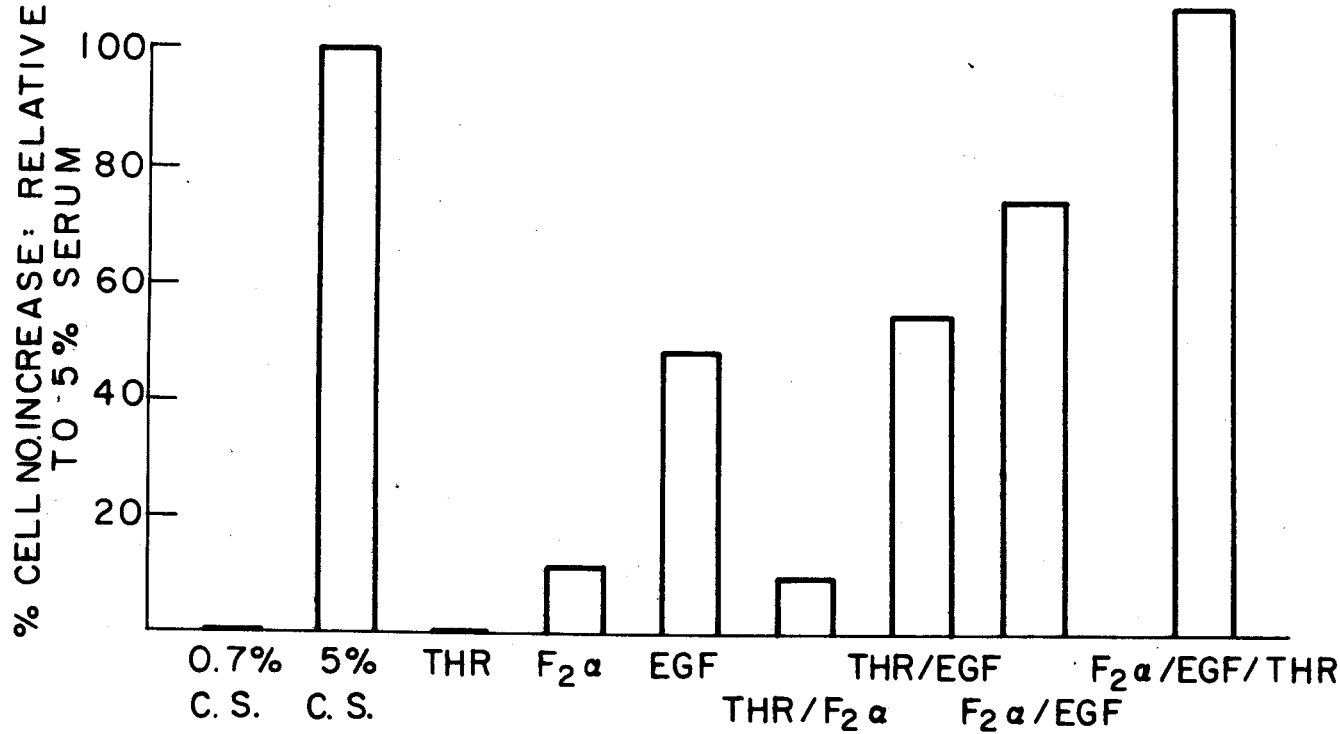
% CELL NO. INCREASE: RELATIVE TO 5% SERUM
100
80
60
40
20
0.7% C. S.
5% C. S.
THR
$F_2\alpha$
EGF
THR/$F_2\alpha$
THR/EGF
$F_2\alpha$/EGF
$F_2\alpha$/EGF/THR

Fig. 2. Potentiation of cell growth of B77-3T3 mouse fibroblasts by thrombin in the presence of other co-mitogens. EGF=epidermal growth factor (0.6 ng/ml) Thr=thrombin (0.2 μg/ml), F2α= prostaglandin F2α (0.2 μg/ml), C.S. = calf serum.

a concentration of 0.2 μg/ml to the B77-3T3 cells results in an increase of only 10% of that of 5% serum. Combination of thrombin with either F2α or EGF does not yield a further stimulation of cell division. However, combinations of F2α and EGF show slightly more than the additive effects of either alone, but in the presence of all three factors, F2α, EGF and thrombin, a response greater than that evoked with 5% serum is obtained. Therefore, it is possible to demonstrate a role for thrombin provided that other essential factors are present. This potentiation for thrombin cannot be demonstrated, however, at the higher saturating concentrations of EGF. Similar synergistic effects of EGF and arginine esterase (or thrombin) on the stimulation of DNA synthesis in an explant of human foreskin has been independently observed by Lembach (36).

The Role of Surface Proteins in Cell Proliferation as Studied with Thrombin and Other Proteases:

As stated in the introductory comments, special attention has centered on an iodinatable cell surface protein of 250,000 daltons (250K) of chick embryo fibroblasts, since it is removed by trypsin during mitogenic stimulation and is lost by some transformed cells. We have attempted to provide further evidence about the role of this protein in mitogenesis by use of thrombin (37). Thrombin is ideally suited for this type of investigation because of its mitogenicity and because of its great specificity as demonstrated by the limited number of peptide bonds it can split. In addition to trypsin and thrombin, several other proteolytic enzymes including bromelin, ficin, chymotrypsin, subtilisin, pronase, and α-protease, were tested both for their capacity to stimulate cell proliferation (or DNA synthesis) and to cleave iodinatable cell surface proteins of chick embryo fibroblasts. Our first efforts centered around an attempt to establish a correlation between mitogenicity and cell surface composition after protease treatment. Two comparisons stood out as particularly revealing, namely, that thrombin could stimulate cells to divide, yet did not cleave the 250K