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This report summarizes a series of studies undertaken to determine if angiogenic molecules isolated from normal tissues and wound fluid could accelerate wound repair. Angiogenesis (new blood vessel formation) is a critical component of many normal and pathologic processes, including wound healing, thus leading to the hypothesis that it angiogenesis during wound repair was increased, then the wound would heal faster. Our first set of experiments completed the purification of the major lipid angiogenic molecule secreted by differentiating adipocytes, 1-butyryl-glycerol. We also determined that adipocytes secrete Vascular Endothelial Growth Factor, a peptide which specifically stimulates endothelial cell proliferation and migration in cell culture, and stimulates angiogenesis in vivo. Synergy between the angiogenic activity of 1-butyryl-glycerol and prostanoids was found, suggesting that multiple signals may be necessary to modulate the angiogenic response in physiologic or pathophysiologic settings. When 1-butyryl-glycerol was tested in a diabetic rat model for wound healing, it was able to significantly accelerate the rate of wound healing in 2 out of 5 experiments. While the lack of reproducibility needs to be resolved, the very positive nature of some of the experiments suggests that 1-butyryl-glycerol may possess clinically useful wound healing capacity.

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John J Castellot Jr 2/14/95

PI - Signature Date

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INTRODUCTION

In this project, we have carried out experiments which have examined the cellular and molecular mechanisms controlling new blood vessel formation during normal tissue development and wound healing. The growth of new blood vessels is an integral part of many important biological processes, including neoplasia, psoriasis, ocular diseases, the oestrus cycle, embryogenesis, and the inflammatory-reparative response. Indeed, angiogenesis is required for the development of any new tissue, whether normal or pathological, and thus represents a potential control point in regulating these processes.

Blood vessels form during embryonic development when islands of primitive endothelial cells arising from the mesenchyme give rise to the blood capillaries. Later in development and in the adult organism, new capillaries are formed from pre-existing vascular beds (1). Regardless of whether the neovascularization is normal or pathological, it consists of at least three readily distinguishable processes involving endothelial cells, each of which can undoubtedly be broken down into multiple, more specific events: 1) changes in attachment to the extracellular matrix and to each other, probably involving the action of proteases (2,3), 2) migration towards the tissue being vascularized (4), and 3) proliferation to provide cells for the new vessels (4,5).

Despite the critical importance of angiogenesis, three major gaps existed in our knowledge of neovascularization mechanisms during wound repair at the inception of this project:

1) the cellular, molecular, and biochemical interactions between granulation tissue and blood vessels during wound healing was virtually unknown,

2) the biochemical nature of the factor(s) responsible for stimulating neovascularization during both normal tissue morphogenesis and wound repair had not been fully characterized, and

3) current attempts to accelerate the rapidity and quality of wound healing with angiogenesis-stimulating molecules had been largely unsuccessful.

This project has provided information that will address these important areas, especially the latter two areas.

Sources of Angiogenic Molecules. A wide variety of cells, tissues, and fluids have been shown to possess angiogenesis-stimulating activity in vivo, and the list continues to grow (see refs. 6,7 for reviews). Most of these, like tumor cells (8), lymphocytes (9), endothelium (10), mast cells (11 12), neutrophils (13), platelets (14,15), retina (16), synovial fluid (17), and vitreous humor (18), are probably related to pathological or abnormal conditions such as cancer, diabetic retinopathy, or inflammation. At least three actively developing adult tissues which stimulate angiogenesis in vivo and which probably represent neovascularization of the

type seen during normal histogenesis have been reported: corpus luteum (19), subblastemal tissue of regenerating deer antler (20), and omentum (21). Neither the biochemical nature nor the cellular sources of the angiogenic activities have been determined. In addition to these sources of angiogenic molecules putatively involved in physiological neovascularization, we have observed that cultured 3T3-adipocytes produce, in a differentiation-dependent manner, factor(s) which stimulate angiogenesis when implanted in vivo (22). The behavior of these cells strongly resembles the angiogenic response to adipocyte differentiation during embryogenesis.

Angiogenesis During Wound Healing. The importance of the intensely vascular nature of the granulation tissue which characterizes wound repair has long been recognized. It is generally agreed that the newly formed blood vessels are necessary for the removal of injurious agents from the wound (eg, bacteria, necrotic cell debris), and that proper closure and re-epithelialization of wound surfaces depends upon the capacity of the local blood supply to provide nutrients and remove waste products. Wound fluid has been shown to stimulate angiogenesis (23), as have macrophages (24), the most abundant cell type present in wound exudates (25). During the past dozen years, it has become evident that the macrophage, and not the granulocyte, plays a critical role in determining the fate of a healing wound (26-28).

Many ultrastructural studies have been carried out on neovascularization during wound healing (see 25 for a review). Until relatively recently, however, there were few reports of studies designed to stimulate wound repair using angiogenic substances, in part because of the unavailability of purified angiogenic molecules (28). There are several recent studies in which the repair of wounds in several species, including humans, has been accelerated. The stimulants included fibroblast growth factor (29-31), transforming growth factor-B (32,33), platelet-derived growth factor (28,34), epidermal growth factor (28,32,34-37), combinations of growth factors (34), growth hormone (38), arachidonate metabolites (39), histamine and serotonin (40), and a live yeast cell derivative (41). It is interesting to note that in many of these studies, increased angiogenesis accompanied the accelerated wound repair. Taken together, the weight of evidence described above strongly suggests that purified angiogenic molecules may be clinically useful stimulators of wound healing.

The importance of angiogenesis in wound healing cannot be overemphasized, as evidenced by the statement made by the eminent pathologist, Dr. Stanley Robbins: "Adequacy of local blood supply may well be the single most important influence in determining the quality and adequacy of the inflammatory-reparative response" (50). Purification of the molecules responsible for angiogenesis should provide a pharmacological basis for therapeutic intervention in wound repair. In particular, the identification of polar lipids and heparin as important molecules in the angiogenic response may lead to methods for the topical application or systemic administration of these substances or their chemically synthesized analogs. This latter possibility is especially intriguing, since it may be possible to develop analogs which maximize angiogenic and wound healing potency without unwanted side effects.

Biochemical Characterization of Angiogenesis Factors. Intense effort has been directed at isolating and identifying the factor(s) responsible for angiogenesis. At least three proteins or families of proteins have been isolated which possess angiogenic activity. A group of heparin-binding growth factors (also called acidic and basic FGF) which stimulate neovascularization have been isolated from several sources (42-44). A 13kD polypeptide with structural and functional homology to RNAse has been purified from human colonic carcinoma cells (45,46), and has been named angiogenin by these investigators. The gene encoding this protein has been isolated, and its biological activities are now beginning to be characterized. Tumor necrosis factor-alpha (TNF-a) was isolated from macrophage conditioned medium and was found to possess angiogenic activity (47). Interestingly, the angiogenic activity from macrophages did not stimulate endothelial cell proliferation in vitro but was chemotactic for these cells.

Previous Studies Related to this Proposal. Because adipocyte differentiation and angiogenesis are tightly coordinated in vivo, we tested the ability of adipocyte secreted products to stimulate neovascularization in vivo (22,48,49). Conditioned medium from 3T3-adipocytes stimulated new blood vessel growth in the chick chorioallantoic membrane (CAM). Control (unconditioned) medium or conditioned medium from preadipocytes did not stimulate neovascularization, even at much higher doses. Thus, the production of the angiogenic activity was strongly dependent on differentiation of the adipocytes. Adipocyte secreted products strongly stimulated protease activity, chemotaxis, and proliferation of endothelial cells. 3T3-preadipocytes produced $< 10\%$ as much of these activities as 3T3-adipocytes, indicating that these in vitro activities are also strongly differentiation-dependent. The mitogenic and chemotactic activities were shown to be specific for vascular endothelium as the target cell.

We also observed that heparin potentiated the angiogenic response to adipocyte-conditioned medium, but had little effect when added to control medium (22,49). When we tested the effect of heparin in our in vitro assays, we found that heparin augmented endothelial cell plasminogen activator activity and chemotaxis, but had no effect on endothelial cell mitogenesis (49). Non-anticoagulant heparin was equally potent and efficacious in the angiogenesis and chemotaxis assays, indicating that the "angiotropic" activity is independent of the anticoagulant activity of heparin. This effect is also specific for heparin compared to other classes of glycosaminoglycans.

Biochemical Analysis. We have isolated the major angiogenic activity from adipocytes in highly purified form. Adipocyte conditioned medium was fractionated by C18 Sep-pak chromatography, followed by thin-layer chromatography (TLC). The angiogenic activity elutes with 50% ethanol, along with many polar lipids. Angiogenic activity could also be recovered in an ether extraction of acidified adipocyte conditioned medium, leading us to suspect that the molecule(s) of interest is (are) polar lipids. When adipose cells are labeled with ^{14}C-acetate, a major differentiation-dependent labeled compound co-elutes with most of the angiogenic activity. When compared with starting conditioned medium, it is apparent that well over half of the starting angiogenic activity is recovered in this fraction. A further

demonstration of the correspondence between this compound and angiogenic activity is shown by utilizing high pressure liquid chromatography (HPLC) to further fractionate the TLC material; angiogenic activity again co-elutes with the ^{14}C-labeled compound. While it is difficult to prove that a material is absolutely pure, we are not able to demonstrate any contaminating biological materials besides this ^{14}C-labeled compound, even when labeling is done with several other precursors to lipids, such as arachidonate or cholesterol.

Our previous work established the particular technical advantages of the adipocyte-endothelial cell and macrophage-endothelial cell systems: a) both the producer (fat cells and macrophages) and target cells (capillary endothelial cells) are identified and available in homogeneous culture, b) the conditions for stimulating secretion of angiogenic molecules can be precisely controlled, and c) molecules which are secreted--a prerequisite for an angiogenic factor--can be specifically collected. The strong stimulations of angiogenic activities coupled with the development of the large-scale chorioallantoic membrane assay should make possible the further biochemical characterization of the angiogenic factors from adipocytes and wound exudates.

It is important to note that the angiogenic activity was fractionated only from the activity which stimulated endothelial cell growth. These studies suggest strongly that blood vessel formation in our system requires separate signals for angiogenesis and mitogenesis, and emphasize the need for caution in selecting just one in vitro assay with which to correlate angiogenic activity. It also underscores the importance of characterizing the endothelial cell mitogenic activity which is also secreted very specifically by adipocytes.

EXPERIMENTAL METHODS AND RESULTS

We have studied the mechanisms controlling angiogenesis during wound healing and other normal tissue formation using a concerted biochemical, pharmacological, and biological approach. Our goals were to:

1) identify and purify angiogenic molecules from adipocytes and from wound fluid,

2) characterize mechanisms regulating the response of blood vessels to angiogenic stimuli,

3) test the ability of angiogenic molecules from adipocytes and wound fluid to accelerate the rate and quality of wound repair in animal models.

As angiogenic molecules were purified by us or identified by others, we assessed their ability to stimulate angiogenesis in vivo as well as protease activity, chemotaxis, and mitogenesis in vitro. Although using multiple assays was more labor-intensive and time-consuming than merely assaying one aspect of the angiogenic response, we felt it to be the only way to obtain results in which we have confidence. All of the methods used are fully documented in the indicated references and in the accompanying Appendix. A more complete explanation of our rationale and procedures can be found in our previous application.

I. CHARACTERIZATION AND PURIFICATION OF ANGIOGENIC MOLECULES

Our initial strategy for characterizing angiogenic molecules was as follows:

Protease Treatment

Destroys Activity — Does Not Destroy Activity

Gel Filtration HPLC — C18 Chromatography — Other Enzyme Treatments

Peptide HPLC (eg, reverse-phase, ion-exchange)	Protein HPLC and/or Classical Protein Chromatography (eg, CM-Sephadex, DEAE-Sephadex, etc)	Lipid HPLC (eg, reverse-phase, silica gel)	Small Organic HPLC (eg, ion-pair, ion exchange)

We first attempted to determine the class of molecules to which adipocyte, wound fluid and macrophage angiogenic molecules belong. Although partial characterizations of wound- and macrophage-derived angiogenic activities suggest that peptide components such as TNF-a may be involved (23,24,47), no studies have examined the possibility that lipid angiogenic molecules are secreted by macrophages or present in wound exudates.

For adipocyte-secreted angiogenic activities, we used cultures of 3T3-F442A adipocytes which were propagated under conditions which promoted almost complete differentiation (48). Cells were allowed to "condition" serum-free DMEM for 6-24 hr, using 1 ml DMEM per 10^6 adipocytes. For wound fluid and macrophage experiments, the starting point for our purification procedures was wound fluid collected from chambers implanted in the backs of rats (51), and macrophage-conditioned medium obtained from primary cultures of rat macrophages (24,52).

Adipocyte Angiogenic Factors. The major angiogenic molecule secreted by adipocytes was highly purified using C18 Sep-pak chromatography, TLC, and HPLC (ref. 72; reprint provided in Appendix A). Because the available biochemical evidence strongly suggested that a large part of the angiogenic activity was lipophilic in nature, we decided to make large quantities of material by utilizing a C18 Sep-pak column step, TLC, followed by C18 HPLC eluted with an acetonitrile gradient. This material was analyzed by mass spectrometry, using gas chromatography-mass spectrometry (electron impact and fast atom bombardment). Since dialysis studies indicated that the molecule was smaller than 1000 daltons, we believed that a determination of structure by this method was feasible. The work was carried out at the NIH-sponsored mass spectroscopy facility at MIT, including their equipment and trained technicians. Our structural analysis demonstrated unambiguously that the material was 1-butyryl-glycerol, a monoacylglycerol which has been previously described as a nutritional supplement, but which heretofore had not been identified as an adipocyte metabolite. Further experiments demonstrated that pre-adipocytes contained little or no detectable amounts of 1-butyryl-glycerol, providing additional corroboration of the likely significance of this molecule as an adipocyte-specific product. Cell biologic characterization of the activity profile of 1-butyryl-glycerol showed that it stimulated angiogenesis in the CAM assay, and also stimulated endothelial cell migration in cell culture studies. Endothelial cell proliferation was not enhanced by 1-butyryl-glycerol. This combination of biochemical and biological evidence clearly established the role of 1-butyryl-glycerol as a specific adipocyte-derived angiogenic molecule.

It is impossible for us to precisely determine how much purified 1-butyryl-glycerol we obtained per liter of conditioned medium. However, using ^{14}C-labeled material and assuming that the angiogenic component is labeled to the same specific activity as fatty acids, we calculated that we recovered approximately 0.2 ug of material per liter. Final proof of the structure for the lipophilic angiogenic factor from wound fluid or macrophages required that the active product from the source be compared with the activity of a chemically synthesized product. This approach has been applied by Corey, Samuelsson, Austen and others to the structural identification of leukotrienes (56-58). These experiments were carried out in

collaboration with Dr. John Fiddes at CalBio, Inc., who provided us with highly purified and characterized synthetic 1-butyryl-glycerol. The biological activity profile of the synthetic molecule matched perfectly the activity profile observed using the 1-butyryl-glycerol purified from adipocyte-conditioned medium (72).

As noted earlier, adipocyte-conditioned medium contained a non-dialyzable, protease-sensitive endothelial cell mitogen. Abraham, Fiddes and others have reported the presence of a highly specific mitogen for vascular endothelial cells which they obtained in native form from the pituitary gland, and in human recombinant form, which has been termed vascular endothelial growth factor (VEGF; ref. 73). We considered the possibility that the mitogen secreted by adipocytes was related to VEGF because the reported biological activity profile of purified VEGF matched closely the activity profile of the mitogenic activity secreted by differentiating fat cells. Using nucleic acid probes for VEGF, we determined that differentiating adipocytes had at least 5-fold higher stable mRNA levels for VEGF than undifferentiated pre-adipocytes (ref. 74). When neutralizing antibodies to VEGF were added to adipocyte-conditioned medium, virtually all of the mitogenic activity was inactivated. These results along with others (see reprint in Appendix) strongly suggested that the mitogenic activity secreted by adipocytes was VEGF, and further suggested the possibility that there might be a synergistic interaction between VEGF and 1-butyryl-glycerol. This possibility has been further explored, as discussed later in this report.

Angiogenic Activity in Wound Fluid and Macrophages. As mentioned above, we collected wound exudate from rats, using stainless steel chambers placed under full-thickness incisions on the backs of Sprague-Dawley rats. By implanting 2 chambers in each rat, we obtained approximately 20 ml of exudate from 10 rats. Although this quantity was much less than we had hoped and limited significantly our ability to purify angiogenic molecules, it did provide sufficient starting material for preliminary characterization of the presence and nature of any angiogenic activity contained therein.

We first determined if the angiogenic activity from wound fluid and macrophages was sensitive to proteases, DNase, RNase, or various glycosaminoglycan-degrading enzymes, using standard methods and treatment conditions for each type of enzyme. Treatment of wound fluid and macrophage-conditioned medium with potent, general proteases was carried out by digestion with immobilized pronase or S. griseus protease over a wide range of concentrations and times (0.1-10 units/ml; 10-120 min). The proteases were removed by centrifugation. Proteolytic digestion was confirmed by examination of ^{35}S-methionine labeled, secreted macrophage proteins on SDS-polyacrylamide gels and autoradiography. Angiogenic activity in vivo and the activity of this material towards endothelial cells in vitro was assayed as described. We detected a loss of mitogenic activity in adipocyte-conditioned medium after protease treatment which was not due to artifactual protease action on the CAM or on the endothelial cells, thus indicating the presence of an essential protein or peptide component. Further purification was attempted using HPLC, but was not successful, most likely owing to the relatively small amount of starting material available.

II. CONTROL OF ANGIOGENESIS VIA BIOCHEMICAL MECHANISMS

The importance of these studies was two-fold. First, virtually nothing was known about the physiological and biochemical mechanisms controlling the response of vascular endothelial cells to angiogenic stimuli, especially polar lipids. Second, modulation of the angiogenic response is a potential mechanism for controlling neovascularization, regardless of whether the angiogenic factors are isolated or characterized. For these studies, we stimulated angiogenesis on the CAM using adipocyte, wound fluid or macrophage-derived products, as described below. Three criteria for angiogenic potential were examined: rapidity, strength, and potency.

Effect of Purified Angiogenic Molecules on Endothelial and Other Cells. The most relevant molecules with which to examine the response of endothelial cells and other vascular cells to angiogenic stimuli are the purified molecules obtained from the biochemical procedures detailed above. To date, this consists of PGE_1, PGE_2, 1-butyryl-glycerol, and VEGF. To this list, we should also add the well-characterized angiogenic peptides of the FGF family. The experiments described below were carried out:

1. Interactions between individual factors which modulate the overall angiogenic response. We mixed purified angiogenic factor(s) in varying proportions. The mixtures were then tested for their ability to affect 1) angiogenesis in vivo, 2) chemotaxis in cultured cells, and 3) mitogenesis. We found that PGEs, especially PGE_2, were capable of acting synergistically with bFGF, and additively with 1-butyryl-glycerol. Interestingly, 1-butyryl-glycerol may have a synergistic interaction with VEGF. There is a technical difficulty in interpreting these experiments which we are attempting to overcome. In our first set of experiments we used human recombinant VEGF, and found that it was unable to synergize with 1-butyryl-glycerol. However, control experiments indicated that this material was biologically inactive in that it could not stimulate angiogenesis on the CAM. When a second batch of VEGF was tested, we did observe synergy between VEGF and 1-butyryl-glycerol in both the CAM and endothelial cell migration assays. Final verification of this synergy awaits completion of experiments with a third preparation of human recombinant VEGF. The results of these experiments, which are being prepared for publication, have allowed us to detect additive and synergistic interactions between the different angiogenic factors, and may help shape strategies for the therapeutic management of wound repair in future animal studies. A copy of the preprint will be sent as soon as it is available.

2. Identification of endothelial cells receptors for angiogenic molecules. There are four criteria which are generally accepted as required characteristics of a prostanoid receptor: a) a high degree of structural specificity for the ligand, b) saturable binding, c) displaceable (reversible) binding, and d) time-dependent binding. We attempted to measure the binding of radiolabeled 1-butyryl-glycerol molecules to monolayer cultures of endothelial cells, using standard methods for polar lipids (59-62). Controls for the binding studies included: 1) determination of the equilibration time required for binding activity, (60,61,63), and 2) examination of the ligand for degradation before it reaches the binding site (62). A time

course (1-180 min) of ligand binding was performed to determine if the binding of 1-butyryl-glycerol to endothelial cells was saturable and time-dependent. Despite repeated efforts to detect either cell surface or intracellular binding sites for 1-butyryl-glycerol, we were unable to do so.

3. Identification of target cells for 1-butyryl-glycerol action. Although the endothelial cell assays for chemotaxis clearly suggested that this cell type was a target for 1-butyryl-glycerol action, we undertook experiments to determine whether other target cells existed for 1-butyryl-glycerol activity. Using a "wrinkle" assay, we obtained evidence that 1-butyryl-glycerol can relax vascular pericytes. In this assay (which is somewhat analogous to the classic fibroblast "contraction" assays done on collagen-coated substrates), the target cell (in this case, pericytes) were plated on culture dishes coated with a deformable polymer. Various agents were added to the culture medium, and the effect on pericyte contraction or relaxation was monitored by observing how "wrinkled" the polymer became. The more relaxed the cells, the less wrinkled the polymer. These observations suggested that the vascular pericyte may be a target cell for 1-butyryl-glycerol action, especially with regard to its effects on blood flow.

III. ROLE OF VEGF IN ANGIOGENESIS

We undertook a series of experiments to determine the role of VEGF in angiogenesis, based on the following observations: 1) up-regulated VEGF synthesis in differentiating fat cells, 2) antibodies to VEGF drastically reduced the mitogenic activity in adipocyte-conditioned medium, and 3) VEGF can synergize with 1-butyryl-glycerol to augment the angiogenic response.

We prepared two "dominant-negative" VEGF constructs. These DNAs, when co-transfected with a vector expressing VEGF, blocked the production of VEGF. This construct was transfected into tumor cells expressing VEGF to determine if endogenous synthesis could be blocked. We found that the dominant-negative VEGF construct, upon transfection into COS cells along with a VEGF expression vector, down-regulated the production of VEGF. We then carried out a series of experiments to determine if this dominant-negative construct suppressed endogenous VEGF activity in a mouse skin permeability assay. In this assay, a blue dye was injected into the tail vein of a mouse. A small patch of skin was exposed on the back of the mouse by shaving off the fur. When VEGF was injected along with the dye, an increase in the blue color of the skin (indicating an increase in capillary permeability to the dye) was observed. When the dominant-negative mutant VEGF was used, the increase in permeability was not seen.

We have carried out a molecular characterization of the dominant negative VEGF mutations. In particular, mutations in cysteine 101 in murine VEGF had inhibitory effects when expressed along with wild-type VEGF in the same cell. Specifically, inhibition was seen in both mitogenesis assays and permeability assays. In the mitogenesis assays, expression of

cys^{101}-VEGF in endothelial cells strongly inhibited their growth rate. Permeability was assessed by the mouse tail vein injection assay. In the presence of VEGF, an increase in permeability was observed; however, this increased permeability was largely blocked when cys^{101}-VEGF was co-injected with the active protein. Our interpretation of the available data suggests that VEGF protein secretion (rather than synthesis or degradation) is interrupted by the mutation.

IV. MODULATION OF WOUND REPAIR

The critical importance of new blood vessels to the quality and rapidity of wound repair makes it very likely that angiogenesis-stimulating molecules can accelerate this process. The goal of the wound repair studies was to find angiogenic and angiotropic molecules, or combinations thereof, which speed up the rate of wound repair while maintaining or improving the quality of wound healing. Many different models in several species have been used to study wound healing, including linear incisions with primary and/or secondary closure (cf. 31,64-68), open wounds made with a punch or scalpel (cf. 26,69,70), and sponge or stainless steel chamber implants (29,36-38,51). Each of these models has its particular advantages and weak points. For our studies, we used the polyvinyl alcohol foam sponge implant procedure described by Davidson and co-workers (29,36). Briefly, 400-450g male db/db rats were maintained in a specific pathogen-free environment. This rat develops diabetes and has impaired wound repair as a result. This model was chosen because it was thought that a normal rat may already have near-optimal rates of wound repair, and that detecting differences in the quality and rate of wound healing would be facilitated in the diabetic rat model. After anesthesia, a 2cm incision was made on the ventral side and four sterile disc-shaped sponges (2mm thick, 10mm diameter) were implanted in four discrete pockets beneath the subcutaneous fascia against the panniculus carnosus and closed with a wound clip. Four days after implantation, the sponges were injected with either a test substance or a buffer control (2 sponges each per animal). At 0, 4, 8, 12, 16, and 20 days after injection, sponges were removed from 3 animals, fixed in formalin and prepared for histological examination. Sponges were scored blind by two investigators on a scale of 0-4 to indicate the degree of development of granulation tissue, where a score of 4 indicates that the entire diameter of the field is filled with well-organized granulation tissue, including fibroblasts, collagen, and blood vessels.

The advantages to the sponge implant model are numerous: It uses a small animal, requires relatively low amounts of test material, and is thus very cost-effective. Large numbers of samples can be tested with both contralateral and systemic controls within the same animal. The inflammatory-reparative response is quantifiable with great statistical confidence in the results, making this system particularly good for screening purposes. It is especially appropriate for examining the early stages of granulation tissue formation, including the angiogenic response. However, this model also has certain limitations. For example, parameters such as the tensile strength of the healed tissue or the rate of closure of a wound

are not measured. This model also has not been used to study the later stages of wound repair, eg, the fibrotic or remodeling phases.

Several sets of experiments were performed using 1-butyryl-glycerol as the experimental drug. These studies were carried out in collaboration with Dr. Corine Klingbeil at CalBio, Inc. Baseline studies indicated that less than 1 ug of recombinant bFGF per sponge elicited a maximal response, which was a reduction in healing time from 21 days to 14 days. In the experiments using 1-butyryl-glycerol, it was tested alone and in combination with bFGF. bFGF was used at its optimal concentration (1 ug/sponge) and a sub-optimal concentration (0.1 ug/sponge). In two of the 5 experiments, 1-butyryl-glycerol was able to substantially reduce the time required to achieve wound closure. In one experiment, the monoacylglycerol was able to reduce healing time to 11 days in the presence of sub-optimal levels of bFGF, and to 13 days when used alone. Unfortunately, there was little or no effect in 3 of the 5 experiments attempted. Because it is exceedingly difficult to generate a "false positive" response in the rate at which a wound heals, it may be reasonably assumed that the two experiments in which accelerated wound repair was observed represent real biological effects of 1-butyryl-glycerol. The negative results are most likely due to technical problems related to dosage regimen, chemical stability, or pharmacokinetic parameters not understood or recognized by the PI.

CONCLUSIONS AND FUTURE DIRECTIONS

There are several areas in which the work done on this project has contributed significantly to the understanding of angiogenesis and its role in modulating wound repair. Identification and characterization at the biochemical and molecular level of the angiogenic molecules secreted by differentiating adipocytes--1-butyryl-glycerol and VEGF--provides an important first step in understanding angiogenesis in normal tissue histogenesis. We have just begun to scratch the surface of characterizing the angiogenic molecules which are present in wound fluid or may be secreted by macrophages, but these avenues of research appear to be promising ones as well. Finally, the observation that 1-butyryl-glycerol can, at least in some instances, dramatically accelerate the rate of wound repair in diabetic rats provides a potentially link between stimulation of angiogenesis and a clinically important endpoint. Because the rate-limiting step in discharging many post-surgical patients from the hospital and returning them to active duty is rate at which their surgical incision heals, shortening this process by even a small increment (ie, 1 day) would have an enormous health and economic impact. There are a number of specific areas which deserve close attention in the near future, and these are delineated below.

We have found that mutations in cysteine 101 in murine VEGF have inhibitory effects when expressed along with wild-type VEGF in the same cell. This inhibitory activity was found in both mitogenesis assays and permeability assays. One of the next steps should be the task of making transgenic constructs that will specifically target the dominant-negative mutants of VEGF to fat cells in the mouse. This will test the hypothesis that VEGF is an important regulator of tissue development. Subsequent gene therapy experiments could be done to see if VEGF could effectively accelerate angiogenesis and therefore stimulate wound repair and resolution of skin lesions.

Despite our inability to demonstrate the presence of either cell surface or intracellular 1-butyryl-glycerol receptors, further experimentation is still indicated. Perhaps preparing the highest specific activity radiolabeled 1-butyryl-glycerol possible would aid in the search for 1-butyryl-glycerol receptors. We are also considering the possibility that the endothelial cell may not be the primary target for 1-butyryl-glycerol and have begun to at other potential target cells.

With respect to the most exciting results we obtained from a potential clinical standpoint, the PI is aware that different types of wounds heal at inherently different rates and with different degrees of restored function (eg, head wounds heal faster than foot wounds, gastric wounds heal faster than colonic ones, skin wounds usually become hypertrophic while muscle wounds generally do not, etc). Thus, certain animal models may more adequately represent certain types of wounds than the sponge implant model. With this in mind, it may be useful to determine if promising results with this model can be applied to other wound healing systems. The partial thickness linear incision wound system developed in the small domestic pig by Eaglstein and co-workers (31,83-85,88) would be an especially interesting

model in which to test promising molecules or combinations revealed by the sponge implant system. This has the advantage of using a tight-skinned animal with an integument more similar to human, and should also permit quantitative assessment of parameters such as tensile strength and rate of closure. It is a well-characterized model in which statistical significance can be achieved with as few as five animals per experimental group, an important consideration in any larger animal model system in today's higher-cost animal care environment. Finally, it would seem imperative to carry out experiments using the sponge implant model in the db/db rat to determine the parameters required to consistently produce an accelerated rate of wound repair.

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ACRONYMS AND SYMBOLS

CalBio	California Biotechnology, Inc.
CAM	chorioallantoic membrane
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
HPLC	high-pressure liquid chromatography
PG	prostaglandin
PI	Principal Investigator
TLC	thin-layer chromatography
TNF-α	tumor necrosis factor-α

APPENDIX

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1-Butyryl-Glycerol: A Novel Angiogenesis Factor Secreted by Differentiating Adipocytes

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Summary

Differentiation of adipocytes is accompanied by secretion of molecules stimulating angiogenesis in vivo and endothelial cell growth and motility in vitro. We demonstrate that the angiogenic and motility-stimulating activities secreted by adipocytes are separable from the endothelial cell mitogenic activity by fractionation of adipocyte-conditioned medium. The major differentiation-dependent angiogenic molecule was purified and identified by GCMS as 1-butyryl-glycerol (monobutyrin). Monobutyrin levels increase at least 200-fold during adipocyte differentiation and represent a major fraction of the total angiogenic activity. Synthetic monobutyrin shows the same spectrum of biological activities as the adipocyte-derived factor: stimulation of angiogenesis in vivo and microvascular endothelial cell motility in vitro, with no effect on endothelial cell proliferation. Angiogenesis is stimulated at doses as low as 20 pg when tested in the chick chorioallantoic membrane assay. These results strongly suggest that monobutyrin is a key regulatory molecule in an angiogenic process linked to normal cellular and tissue development.

Introduction

The formation of new blood vessels, or angiogenesis, is an integral part of many normal and pathological processes, including embryogenesis, inflammation, wound healing, neoplasia, ocular diseases, and rheumatoid arthritis. New blood vessels arise from endothelial cells in preexisting microvascular beds by a complex process that requires proteolytic degradation of basement membrane in the parent vessels, directed migration toward the new site, and proliferation to provide cells for the new vessels (Reich, 1974; Ausprunk and Folkman, 1977; McAuslan and Hoffman, 1979; Rifkin et al., 1984). Angiogenesis factors have been characterized by their ability to promote the formation of new blood vessels in vivo and to stimulate one or more of the following endothelial cell functions in vitro: protease production, motility, and proliferation.

A wide variety of cells and tissues have been shown to possess angiogenesis-stimulating activity (reviewed in Folkman and Klagsbrun, 1987). Isolation and characterization of angiogenesis factors from these sources have revealed a striking diversity in structure and function. One class of angiogenesis factors is polypeptides, including acidic and basic fibroblast growth factors (Moscatelli et al., 1986; Abraham et al., 1986; Vlodavsky et al., 1987; Moscatelli, 1987, 1988), transforming growth factors α and β (Schreiber et al., 1986; Roberts et al., 1986), angiogenin (Fett et al., 1985), and tumor necrosis factor α (Leibovich et al., 1987). Nonpeptide angiogenesis factors constitute a second type of angiogenesis factor and include prostaglandins E_1 and E_2 (PGE_1 and PGE_2; Ben-Ezra, 1978; Ziche et al., 1982; Form and Auerbach, 1983), hyaluronic acid fragments (West et al., 1985), and nicotinamide (Kull et al., 1987). Other factors, such as heparin (Taylor and Folkman, 1982; Castellot et al., 1982) and copper (McAuslan and Hoffman, 1979; Raju et al., 1984), have been shown to modulate the angiogenic response but are not themselves angiogenic. Additional angiogenic factors have yet to be fully characterized (Banda et al., 1982; Goldsmith et al., 1984). While it is clear that most of these factors are able to stimulate angiogenesis in the rabbit cornea or chick chorioallantoic membrane (CAM) bioassay systems, their developmental and physiological relevance as angiogenesis stimulators remains unclear.

Angiogenesis during normal tissue development is the most common type of neovascularization. Adipocyte differentiation and angiogenesis are tightly coordinated events during embryogenesis, and the newly formed adipose tissue depends on continued angiogenesis for further development (Wasserman, 1965). Since new blood vessels are formed from endothelial cells, and cultured mouse 3T3 adipocytes have most of the attributes of fat cells in vivo (Green, 1978), the interaction between 3T3 adipocytes and cultured endothelial cells provides an excellent model system for the study of developmental angiogenesis. Previous work from our laboratories has shown that cultured 3T3 adipocytes secrete factors that strongly stimulate angiogenesis in vivo and endothelial cell protease production, chemotaxis, and mitogenesis in vitro (Castellot et al., 1980, 1982, 1986). Production of these angiogenesis factors is highly differentiation dependent, since at least 10 times more angiogenic activity is secreted by 3T3 adipocytes than by their undifferentiated precursors. The identity of the molecule(s) responsible for this response has not been determined previously. In this study, we purify a single compound from adipocytes that

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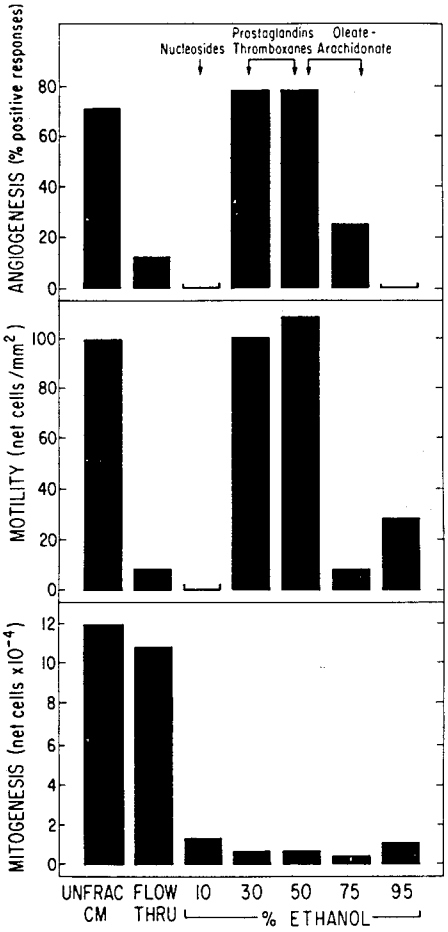

Figure 1. C_{18} Fractionation of Adipocyte-Conditioned Medium

3T3 adipocyte-conditioned medium (10 ml) was applied to a Sep-Pak C_{18} cartridge (Waters Assoc.). Fractions were eluted with the indicated percentages of aqueous ethanol (2 ml), evaporated to dryness, and resuspended for bioassay (as described in Experimental Procedures). Angiogenesis is reported as the percentage of positive samples; at least 20 samples were tested for each fraction. Motility is reported as net cells per mm^2, i.e., the number of cells per mm^2 on a test medium filter minus the number of cells per mm^2 on a control medium filter. Mitogenesis is reported as the net number of cells in test medium minus the number of cells in control medium. The aqueous material that did not bind to the C_{18} column is labeled "flow thru"; "unfrac CM" denotes unfractionated conditioned medium. The elution positions of nucleosides (adenosine, thymidine), fatty acids (arachidonate, oleate), prostaglandins, and thromboxanes are marked with arrows.

contains a major fraction of the total angiogenic and motility-stimulating activities and identify this novel differentiation-dependent angiogenesis factor as 1-butyrylglycerol (monobutyrin).

Results

Separation of Angiogenic and Mitogenic Activities

Preliminary biochemical analysis suggested that the adipocyte-derived angiogenic factors were nonproteinaceous, lipophilic molecules. Reverse-phase chromatography of adipocyte-conditioned medium on Sep-Pak C_{18} columns (Waters Assoc.) separates the mitogenic activity from the angiogenic and motility-stimulating activities (Figure 1). Fractions stimulating angiogenesis on the chick CAM and endothelial cell motility in vitro are retained on the C_{18} column and elute with 30%–50% ethanol. Greater than 80% of the angiogenic and motility-stimulating activities in the unfractionated conditioned medium are recovered in the 30%–50% aqueous ethanol fractions. Unlike the angiogenic components, the endothelial cell mitogen does not bind efficiently to the C_{18} column; greater than 90% of the mitogenic activity is recovered in the flowthrough.

Table 1. Angiogenic Activities (% Positive Responses) of Thin Layer Chromatography Fractions

Fraction Number	Adipocyte-Conditioned Medium			Preadipocyte-Conditioned Medium (Undiluted)
	Undiluted	Diluted 1:1	Diluted 1:49	
1	0			
2	1			
3	14			
4	56	27	5	33
5	77	76	29	5
6	11			
7	0			
8	0			

The combined 30% and 50% ethanol fraction from C18 Sep-Pak cartridges was chromatographed on silica gel thin layer plates in a mobile phase of ethyl acetate–2,2,4-trimethylpentane–acetic acid–water (110:50:20:10, (with fraction 1 nearest to the origin) and eluted with 95% ethanol; 25% of each eluate was evaporated, resuspended in 0.5 ml of control medium (0.5% fetal calf serum in DMEM) containing 0.2% ethanol, and tested for angiogenic activity. Samples were used at a concentration equivalent to the starting conditioned medium (assuming no loss during purification), or were further diluted with control medium. Control medium alone yielded 11% positives, and this value was subtracted from all experimental values to yield the data shown above. Unfractionated conditioned medium gave the following % positive responses: adipocyte 78%, preadipocyte 2%; adipocyte-conditioned medium diluted 1:49 yielded 49% positive responses. All data are derived from at least 40 samples.

Further fractionation of the angiogenic activity was carried out by thin-layer chromatography. Material eluted from the C_{18} Sep-Pak column with 30%–50% ethanol was applied to a silica gel thin-layer plate and developed. The lane was divided into eight fractions, which were scraped, eluted with ethanol, and assayed for angiogenic activity (Table 1). Undiluted thin-layer chromatography fractions 4 and 5 (TLC4 and TLC5) were found to have considerable angiogenic activity. However, comparison of the angiogenic activity in these fractions by dilution experiments indicates that TLC5 contains approximately 5–8 times more angiogenic activity than TLC4 and accounts for most of the angiogenic activity secreted by 3T3 adipocytes (Table 1).

Differentiation Dependence of Angiogenic Factors

The effect of adipose differentiation on the level of angiogenic activity in TLC4 and TLC5 was analyzed (Table 1). Adipocyte TLC4 has only about 50% more angiogenic activity than the comparable 3T3 preadipocyte TLC4 fraction. In contrast, the activity in TLC5 appears strongly

differentiation dependent. Quantitatively, there is an increase in TLC5 activity during differentiation from 5% to 77% net positives, which mirrors the increase in angiogenic activity of unfractionated conditioned medium from 3T3 preadipocytes compared with adipocytes (2% vs. 78% net positives in CAM assays; see legend to Table 1). It is possible that 3T3 preadipocytes are unable to synthesize as much TLC5 activity as 3T3 adipocytes, because of differences in the serum used to culture the cells (calf vs. fetal calf). However, nondifferentiating 3T3-C2 cells grown in medium identical to that used for 3T3 adipocytes have TLC5 activity levels comparable to preadipocytes (6% net positives). Thus, production of the most active angiogenic fraction (TLC5) appears to be tightly linked to adipocyte differentiation.

Analysis of endothelial cell motility-stimulating activity also showed that the most active differentiation-dependent chemoattractant was contained in TLC5, although other fractions have significant activity (data not shown).

Identification of the Major Angiogenic Factor

To avoid the extremely difficult task of purifying the angiogenic factor(s) to homogeneity via the bioassays, we first sought to identify molecules that had the biochemical profile of the active molecule(s) that had emerged from this partial purification: lipid-like, differentiation dependent, and migration in TLC5 after ethanol elution from Sep-Pak columns. Cellular lipids were labeled metabolically with [^{14}C]arachidonate or [^{14}C]acetate. Arachidonate is incorporated predominantly into lipids of the prostaglandin and leukotriene families, while acetate is incorporated into several broad classes of lipids. A single major molecular species that labeled with acetate migrated in the adipocyte TLC5 fraction (Figure 2, right, lane F; region marked with arrows), and this band was not observed in material derived from nondifferentiating 3T3-C2 cells (lane C). When this acetate-labeled band was excised and directly assayed for angiogenic activity, it was found to contain most (88%) of the activity present in adipocyte TLC5 (data not shown).

The arachidonate-labeled sample displayed no prominent species in the adipocyte TLC5 fraction (Figure 2, left, lane F), but did show a band migrating just below the TLC5 region in what would be the TLC4 fraction. We identified this arachidonate metabolite, which showed no dependence on differentiation (compare lanes C and F in Figure 2, left), as PGE_2 and showed by radioimmunoassay that the levels of PGE being produced were sufficient to account for most of the angiogenic activity observed in the TLC4 fraction.

After excision of the [^{14}C]acetate-labeled band in TLC5 derived from adipocytes, further purification was carried out by reverse-phase C_{18} high pressure liquid chromatography (HPLC) with an aqueous isopropanol gradient (Figure 3). A single peak labeled with ^{14}C emerged from the column; the material in this peak did not absorb light at 254 or 280 nm. Overall recovery of ^{14}C radioactivity in this peak was 90%. We scaled up the purification protocol and processed 100 ml of 3T3 adipocyte-conditioned medium to obtain enough material to analyze by gas chromatography and mass spectrometry (GCMS).

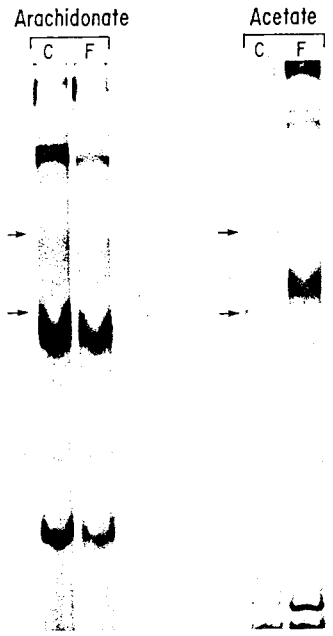

Figure 2. Thin-Layer Chromatography of Secreted Products

Cells were labeled in the absence of serum for 16 hr with [1-^{14}C]arachidonate (59.6 mCi/mmol; NEN) or [1,2-^{14}C]acetate (55.2 mCi/mmol; NEN) using 10 μCi/ml (50 μCi total). The labeled conditioned medium was loaded onto Sep-Pak C_{18} cartridges, washed with water, and eluted with ethanol. The 30%–50% ethanol fractions were dried, resuspended in 100 μl of 95% ethanol, loaded onto silica gel thin-layer plates, and developed as described in Experimental Procedures. ^{14}C-labeled metabolites were visualized by autoradiography or fluorography with En^{3}Hance (NEN). Secreted products are derived from either undifferentiated 3T3-C2 cells (lanes C) or 3T3-F442A adipocyte cultures (lanes F).

This labeled HPLC fraction was converted to the trimethylsilyl derivative and analyzed by gas chromatography (GC) (Figure 4). The most abundant molecular species derived from the adipocyte fraction eluted from the column at 8.49 min (Figure 4B), and this species was virtually absent from a parallel purification carried out on conditioned medium from 3T3 preadipocytes (Figure 4A). We estimate the quantitative increase of this molecule during differentiation to be at least 200-fold. When another portion of the trimethylsilylated fraction was subjected to GCMS, the 8.49 min peak produced the mass spectrum shown in Figure 5 (top). Interpretation of this spectrum indicated that this compound is 1-butyryl-glycerol ($HOCH_2$-$CH[OH]CH_2O_2CC_3H_7$; monobutyrin). Chemically synthesized monobutyrin gave GC and GCMS patterns identical to the adipocyte-derived ^{14}C-labeled molecule (Figure 5, bottom).

Further confirmation of the identity of the acetate-labeled band in TLC5 as monobutyrin was obtained in the following way. First, synthetic monobutyrin comigrates with the biologically active ^{14}C-labeled molecule in three different thin-layer chromatography solvent systems as detailed in Experimental Procedures. Second, treatment of the ^{14}C-labeled molecule with NaOH or prolonged stor-

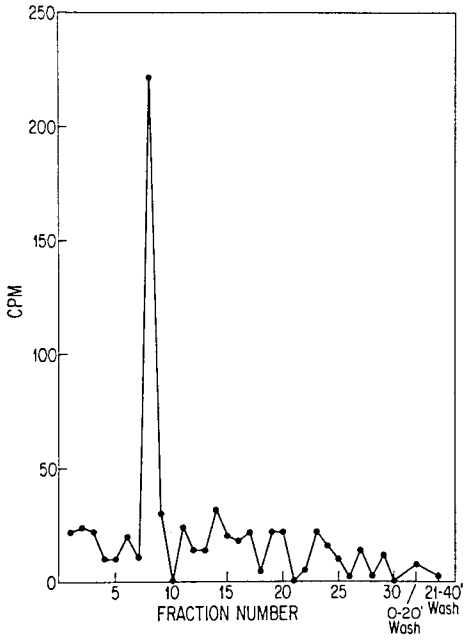

Figure 3. HPLC of [^{14}C]Acetate-Labeled Band TLC5 from 3T3 Adipocytes

Conditioned medium (100 ml) containing 5 ml of [^{14}C]acetate-labeled conditioned medium was fractionated by C_{18} Sep-Pak and thin-layer chromatography as described in Figures 1 and 2. The ^{14}C-labeled band in TLC5 was visualized by autoradiography, excised, and eluted in 95% ethanol for 16 hr. A sample treated identically was prepared from 3T3 preadipocytes in parallel. The eluted material was dried, resuspended in 100 μl of water, and subjected to HPLC on a C_{18} column (Vydak 218.TP with Waters Bondpak C_{18} Guard-Pak) using a 0%–15% aqueous isopropanol gradient over 30 min (1 ml/min flow rate). The column was then washed with 60% isopropanol for 40 min. One milliliter fractions were collected, and 10 μl of each fraction was removed and counted by liquid scintillation; 24,600 ^{14}C cpm from the adipocytes was loaded onto the HPLC column, and 22,200 cpm (90%) was recovered in fraction 8.

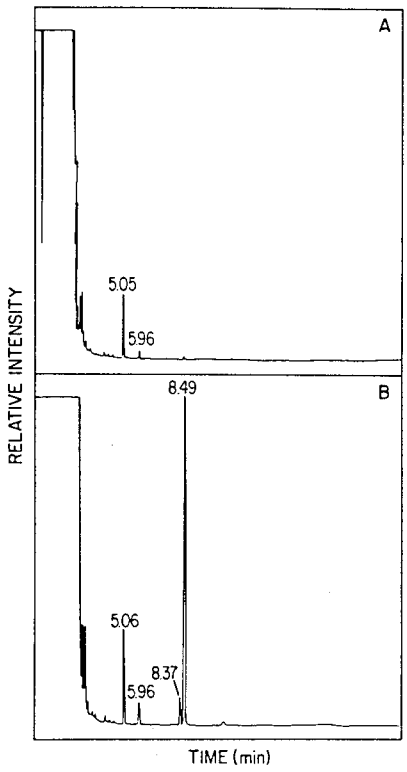

Figure 4. Gas Chromatogram of the HPLC-Purified Angiogenic Fraction

The fraction containing the major ^{14}C-labeled peak derived from adipocytes in HPLC (fraction 8 in Figure 3) and the same fraction from identically prepared preadipocyte-derived material were analyzed by GC after derivitization with BSTFA. (A) Preadipocyte-derived fraction. (B) Adipocyte fraction.

age in water (pH 5.0) releases a single radiolabeled species that comigrates with [^{14}C]butyrate, as predicted from the structure of monobutyrin.

Synthetic Monobutyrin Has the Same Profile of Biological Activities As the Major Angiogenesis Factor

Synthetic monobutyrin was highly purified as described in Experimental Procedures and used in all three angiogenesis-related assays. Monobutyrin was angiogenic in the chick CAM assay at a broad range of concentrations, from 20 pg to 2 μg per pellet, with a maximum response (65% positives) at 20 ng per pellet (Figure 6). Histological examination of positive responses revealed no evidence of frank inflammation. An increase in microvascular endothelial cell motility was also observed across a broad concentration range, 7×10^{-8} M to 7×10^{-3} M, with a maximum at approximately 7×10^{-5} M (Figure 7A). This motility-stimulating activity appears to have specificity for endothelial cells, in that monobutyrin had no effect on the motility of vascular smooth muscle cells or BHK fibroblasts (data not shown). In contrast to these positive results with angiogenesis and motility, no effect of monobutyrin on endothelial cell proliferation was observed across a broad range of concentrations from 7×10^{-9} to 7×10^{-3} M (Figure 7B). This experiment was performed at a variety of serum concentrations and cell densities (1×10^3, 4×10^3, and 2×10^4 cells per cm^2), and no growth stimulation was ever observed. Thus, synthetic monobutyrin has a biological activity profile that is identical to that observed with the adipocyte-derived angiogenesis factor: stimulation of motility and angiogenesis but no direct stimulation of endothelial cell growth.

Based on the assumption that the specific activity of monobutyrin is equal to the specific activity of fatty acid pools in 3T3 adipocytes labeled with [^{14}C]acetate, we estimate that adipocytes secrete approximately 10 μg of monobutyrin per liter of conditioned medium in 16 hr, equivalent to 200 pg per pellet applied in the angiogenesis assays (see Experimental Procedures). Although this calculated level of monobutyrin is clearly approximate, it does suggest that the amount of monobutyrin secreted by fat cells is sufficient to be angiogenic.

Discussion

This work describes the discovery of a novel angiogenic molecule, 1-butyryl-glycerol (monobutyrin). This factor stim-

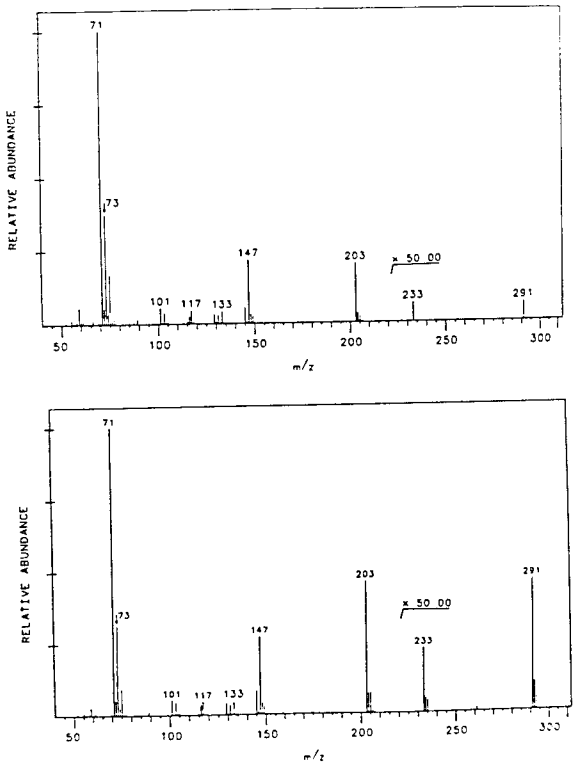

Figure 5. Electron Impact Mass Spectrum of Purified Adipocyte Angiogenic Factor

The top panel shows the mass spectrum of the trimethylsilyl (TMS)-derivitized peak with the retention time of 8.49 min in Figure 4B (derived from adipocytes) and the mass spectrum of the TMS derivative of synthetic DL-1-butyryl-glycerol (bottom panel). The relative abundances in the region from m/z 225–300 have been multiplied by a factor of 50 in these spectra. The molecular weight of 2,3-bis(trimethylsilyl)-1-butyryl-glycerol is 306. As is typical for trimethylsilyl derivatives, the molecular ion (M^+) is absent, and a peak due to (M^+-CH_3) is observed at m/z 291. Loss of either TMS ($Si[CH_3]_3$) group results in the peak at m/z 233. Loss of CH_2OTMS gives m/z 203. Other abundant fragments are $^+CH_2OCOC_3H_7$ at m/z 101 and $C_3H_7OCO^+$ at m/z 71. The ions at m/z 147 and 73 are characteristic of TMS derivatives of diols. The analog prepared with deuterated trimethylsilylating reagent supports this interpretation, as all peaks shifted as predicted.

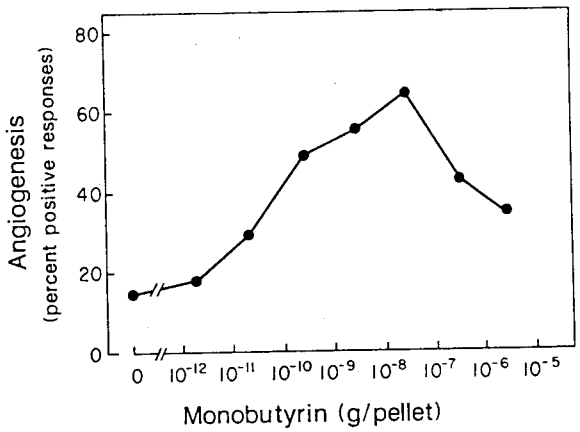

Figure 6. Angiogenic Activity of Monobutyrin

Highly purified synthetic monobutyrin (see Experimental Procedures) was tested in the CAM assay for angiogenesis. Controls contained vehicle alone (DMEM plus 2% fatty acid–free, immunoglobulin-free bovine serum albumin). All SEMs are <6%. The angiogenic responses observed at 20 pg per pellet and higher concentrations are significantly different from control values ($p < .05$ for 20 pg per pellet, $p < .02$ for all higher concentrations).

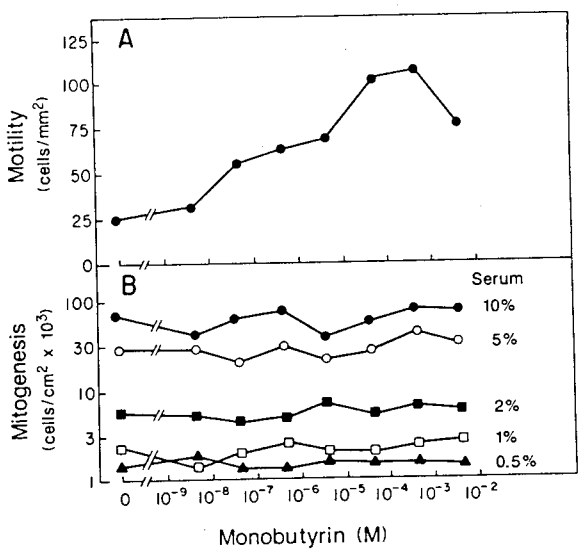

Figure 7. Motility- and Growth-Stimulating Properties of Monobutyrin

Microvascular endothelial cells were treated with monobutyrin at the stated concentrations and assayed for motility (A) or mitogenesis (B) by the methods described in Experimental Procedures. For the motility data presented, all SEMs are <9 cells per mm². The motilities of cells exposed to 6×10^{-8} M and higher concentrations are significantly different from control values ($p < .02$). For the mitogenesis data presented, all SEMs are <12% of the value plotted. None of the monobutyrin concentrations yielded statistically significant differences when compared with control values (all p values > 0.2).

ulates angiogenesis in the chick CAM assay potently (20 pg) and without any detectable effects on endothelial cell growth in vitro. Monobutyrin has not been previously recognized (to our knowledge) as a major lipid constituent or as a secretory product of adipose cells or any other cell types. The biochemical pathway leading to the production of monobutyrin is not clear at this time, but it seems likely to involve at least some of the typical enzymes of fatty acid synthesis and acylation. These enzymes exist in most or all cells, although adipocytes have elevated levels of many of the rate-limiting enzymes (Green, 1978). Given the important biological actions of this molecule demonstrated here, the extent and conditions under which other cell types might produce monobutyrin appear worthy of exploration.

Over the past few years a number of other molecules (mostly, but not exclusively, polypeptides) have been shown to have angiogenic activity in in vivo assay systems. Clearly, a major challenge facing this field of study is to determine the biological relevance of these factors in the wide variety of normal and pathological contexts in which blood vessel development takes place. One conceptually simple approach to the question of biological relevance is to try to define both quantitatively and qualitatively the major angiogenic activities that are temporally

associated with a particular developmental process. Adipose tissue, being the major depot of energy stores in higher vertebrates, is highly vascularized, and this vascularization is clearly coupled in time and space to the differentiation of adipose cells during embryological and postnatal development (Wasserman, 1965). In the experiments described here, we demonstrate that a major fraction of the angiogenic activity secreted by adipocytes resides in monobutyrin. Furthermore, like the angiogenic activity itself, the production of monobutyrin is linked tightly to the differentiation process. Thus, these data suggest very strongly that monobutyrin is a key molecule regulating the angiogenesis program during the development of adipose tissue in vivo.

In addition to monobutyrin, adipocytes secrete levels of PGE_1 and PGE_2 that are angiogenic. The secretion of the PGEs is not strongly regulated during adipocyte differentiation. While the angiogenic activity of prostaglandins has been previously reported (Ben-Ezra, 1978; Ziche et al., 1982; Form and Auerbach, 1983), the physiological role that E series prostaglandins play in the neovascularization of specific tissues, including adipose tissue, remains an open question.

Monobutyrin stimulates angiogenesis in vivo and microvascular endothelial cell motility in vitro, but it is not directly mitogenic for endothelial cells. Some precedent for a nonmitogenic angiogenesis factor comes from studies of extracts of wound fluid (Banda et al., 1982). Since angiogenesis must necessarily involve an increase in endothelial cell number, an important question is how this mitogenesis might occur. Angiogenesis is commonly believed to be a multiple-step process that is initiated by the liberation of endothelial cells from existing blood vessels and the directed migration of these endothelial cells toward a source of angiogenic substances (Reich, 1974; Ausprunk and Folkman, 1977; McAuslan and Hoffman, 1979; Rifkin et al., 1984). The initiating events at a cellular level are likely to involve degradation of the extracellular matrices of the blood vessel and loss or loosening of cell–cell contacts in the endothelial layer, followed by chemotaxis. After endothelial cells begin to move through tissue space in a guided fashion, their proliferation may be stimulated by molecules mitogenic for this cell type. These could include known endothelial cell mitogens (e.g., transforming growth factor α or fibroblast growth factor) that may be present endogenously in tissue space and may also include the mitogenic factor secreted by adipose cells described here that is not angiogenic by itself.

Monobutyrin is a monoacylglycerol that has not been previously reported to have angiogenic activity nor any other pharmacological actions. It has been used in nutritional studies where it was shown to be an effective dietary energy source and has also been used as a food preservative and solubilizing agent (Bancher et al., 1973; Birkhahn et al., 1977; Haas et al., 1984). In addition, monobutyrin can act as a substrate for bile salt–activated triacylglycerol lipase isolated from human milk (Wang, 1981). The relatively low levels of monobutyrin that can stimulate angiogenesis in the chick CAM assay (20 pg per pellet) and endothelial cell motility in vitro (7×10^{-8} M) suggest that it works via an intracellular or cell surface receptor–mediated action. While monobutyrin itself has not been described as a receptor-mediated ligand, there is ample precedent for other mono- and diacylglycerides having a wide variety of actions through interactions with protein kinase C or diacylglycerol kinase (Bell, 1986; Wright et al., 1988). Whether these or similar effector systems mediate the actions of monobutyrin in endothelial cells remains to be determined.

Experimental Procedures

Cell Culture

All cells were cultured at 37°C in a humidified, 5% CO_2–95% air atmosphere. All growth media contained 4 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Capillary endothelial cells were isolated and cultured essentially as described by Wagner and Matthews (1975) from rat epididymal fat pads. The tissue was minced, digested with collagenase, and centrifuged (600 × g, 5 min, 25°C). The pellet, containing only blood vessels and stromal cells, was sieved through a 50 μm pore screen to separate small blood vessels, which were retained on the screen from stromal elements. The small vessels were placed into tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and nutrient mix K1 (Taub and Sato, 1980). The endothelial identity of each resulting culture was verified by the distinctive morphology and by the presence of angiotensin-converting enzyme (Jaffe, 1984).

3T3-F442A and 3T3-C2 cells were grown as described (Green and Kehinde, 1976) in DMEM containing 10% fetal calf serum and 0.5 μg/ml insulin. To minimize adipose conversion, 3T3-F442A stocks were grown in DMEM containing 9% cat serum, 0.5% calf serum (Kuri-Harcuch and Green, 1978).

Conditioned Medium

Conditioned medium was collected from cells grown and maintained for 5–7 days after confluence unless otherwise noted. Cells were washed three times with DMEM, and 5 ml of DMEM was added to each 100 mm culture dish. After 16–24 hr, the conditioned medium was removed, flushed with nitrogen, and stored in the dark at −70°C. Under these conditions, the activities in conditioned media are stable for at least 6 months.

Angiogenesis

Angiogenic activity was assayed on the chick CAM using a modification of the shell-less embryo technique developed by Dunn et al. (1981). The embryo is suspended in a "sling" made of plastic wrap (Grand Union) held in a styrofoam drinking cup with a rubber band. Chicken eggs 66–78 hr after fertilization were cracked into the plastic wrap sling, covered with a sterile plastic top from a 100 mm culture dish, and placed in a humidified 38°C incubator. Eight days after fertilization, 40 μl agarose pellets containing test substances were placed on the CAM, which occupies an area of 30–40 cm^2 at this stage. Pellets were prepared by rapidly combining 20 μl of 4% aqueous low melting point agarose (Sigma type IX) with an equal volume of test substance at 37°C, thereby avoiding any abnormal heating of the test material. The pellets were placed at 4°C for several minutes to solidify. This technique results in >50% survival of the embryos, allows direct visual and photographic monitoring of the experiment, and permits multiple samples to be tested on a single egg. Scoring was done in a single blind fashion on days 3–5 after addition of test samples. Histological sections of positive responses were routinely prepared and examined for the presence of inflammatory cells, since inflammation of the CAM could also result in a positive response. The range of positive responses seen with known angiogenic factors (e.g., fibroblast growth factor) varies from 50%–80% in a typical experiment.

Motility

Migration assays were performed using modified Boyden blind well chambers (Neuro Probe) as previously described (Postlethwaite et al., 1976; Grotendorst et al., 1981; Castellot et al., 1982). The test sub-

stance, containing 0.5% fetal calf serum, was placed in the bottom chamber. A gelatin- and fibronectin-coated filter was inserted, and cells in DMEM containing 0.5% fetal calf serum were added to the top chamber. The chambers were incubated for 120–180 min at 37°C. The filters were removed and fixed in methanol, and cells on the upper filter surface were carefully removed. After staining with Giemsa, the number of cells on the lower filter surface was determined by counting five to ten high power fields (430×) in a light microscope.

Mitogenesis

The mitogenic activity in conditioned medium was assayed as previously described (Castellot et al., 1980). Aortic or microvascular endothelial cells between passages 3 and 10 were plated into 24-well culture dishes. Mitogenic activity was determined by measuring cell number with a Coulter counter after 3–4 days in culture.

C_{18} Fractionation

Sep-Pak C_{18} cartridges (Waters Assoc.) were used to fractionate conditioned medium. Twenty milliliters of conditioned medium was applied sequentially to two columns. Fractions were eluted with increasing concentrations of ethanol in water: 2 ml each of 10%, 30%, 50%, 75%, and 95% ethanol were used. Fractions were evaporated to dryness and resuspended either in DMEM containing 0.2% ethanol for bioassay or in 95% ethanol for further fractionation by thin-layer chromatography. Samples were stored under nitrogen in the dark at −80°C until assayed.

Thin-Layer Chromatography

Thin-layer chromatography was carried out on channeled silica plates (20 × 20 cm) with preabsorbant zone (J. T. Baker Co.). The mobile phase was the organic phase of ethyl acetate–isooctane–acetic acid–water (110:50:20:10, by volume; Salmon and Flower, 1982) unless otherwise stated. The plates were developed and then air dried in the dark, and results were visualized by autoradiography. [^{14}C]monobutyrin comigrates with the extracted [^{14}C]acetate-labeled band TLC5 using the following mobile phases (v/v): ethyl acetate–isooctane–acetic acid–water (110:50:20:10), R_f = 0.48; ethyl acetate–isooctane–water (110:50:10), R_f = 0.30; ethyl acetate–chloroform (60:40), R_f = 0.24; benzene–acetone (75:25), R_f = 0.19.

GCMS

GC was carried out with a Varian model 2700 gas chromatograph (Varian Assoc., Palo Alto, CA) equipped with an on-column injector (J & W Scientific, Folsom, CA), a flame ionization detector, and a 30 m DB-5 fused silica capillary column (0.25 mm film thickness) using helium (18 psi) as the carrier gas. After a hold of 2 min at 70°C, the oven temperature was raised to 310°C at the rate of 10°C per min. The flame ionization detector was held at 310°C. Chromatograms were recorded with a Varian model 4290 integrating recorder. For GCMS, a similarly equipped Varian gas chromatograph was connected to a Finnigan/MAT 212 mass spectrometer by butting the end of the column to a fused silica capillary transfer line, which was maintained at a temperature of 310°C. The ion source temperature was held at 200°C, and the source pressure was approximately 4×10^{-6} mbar. The ionization voltage was 70 V, and the emission current was 1.0 mA. The mass range from 50 to 500 amu was scanned at a rate of 1.1 s/decade (with a 0.8 s interscan time) at a resolution of approximately 1:800. Data were recorded and processed with a Finnigan/MAT SS200 data system. When samples were introduced directly into the ion source of the mass spectrometer, the same instrument parameters were used, except that the ion source pressure was approximately 8×10^{-8} mbar and the magnet was scanned at a rate of 3.3 s/decade. The sample was placed into a small quartz crucible (Scientific Instrument Services Inc., Ringoes, NJ) in the direct insertion probe. Mass spectra were recorded while the crucible was heated to 400°C at a rate of 2°C/s.

Preparation of Trimethylsilyl Derivatives

Samples were dissolved in 1 ml of ethyl acetate, transferred to a 1 ml screw-capped reaction vial (Pierce, Rockford, IL), and dried under a gentle stream of nitrogen. Approximately 100 μl of N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA; Pierce) was added to the dried sample. The vial was sealed with a Teflon-lined cap and heated at 70°C in a heating block for 15 min. Following this, 1–2 μl aliquots were removed for GC and GCMS analyses. For preparation of deuterated trimethylsilyl derivatives, the sample was treated as above with the exception that $^2H_9$ BSTFA (Pierce) was used as the derivatizing reagent.

Purification of Monobutyrin

DL-monobutyrin was purified using the following procedure: practical grade monobutyrin (Kodak) was subjected to vacuum distillation and the 137°C to 138°C distillate (3 mm Hg) recovered. This distillate was run through an open silica gel column, and three peaks were resolved. The material in the first peak was recrystallized from ether at −70°C; analysis of this material by GC and fast atom bombardment mass spectrometry identified it as >99% pure 1-butyryl-glycerol (monobutyrin).

Alkaline Hydrolysis of Adipocyte-Derived Monobutyrin

^{14}C-labeled TLC5 was resuspended in 0.1 N NaOH for 30 min at 23°C. An equal volume of 0.1 N HCl was added to neutralize and stop the reaction, and the mixture was evaporated to dryness in a Speed-Vac (Savant, Bridgeport, NY). The dried sample was resuspended in 50 μl of 95% ethanol and chromatographed as described in the thin-layer chromatography section.

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Biosynthetic Regulation of Monobutyrin, An Adipocyte-Secreted Lipid With Angiogenic Activity

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Running Title

Monobutyrin Regulation in Adipocytes

Summary

1-butyryl-glycerol (monobutyrin) is a novel angiogenic compound that is synthesized and secreted during the differentiaton of 3T3-F442A preadipocytes into adipocytes. To study the regulation of monobutyrin biosynthesis, we have developed an assay utilizing the glycerol kinase enzyme from *Cellulomonas* to quantitate the levels of this compound in cell-conditioned media. Analysis of several cultured cell types, including tumor cell lines, indicated that monobutyrin production is detectable only from adipocytes, reaching a steady state concentration of approximately 1.0 μM in conditioned media. Monobutyrin synthesis was demonstrated *in vitro* using [^{14}C]butyryl-CoA with total homogenate or particulate fractions from adipocytes. Similar fractions from non-adipocyte cell lines failed to synthesize monobutyrin. This biosynthetic activity was shown to be distinct by substrate competition studies from the microsomal *sn*-glycerol-3-phosphate acyltransferase, whose activity is known to increase during adipocyte differentiation. The production of monobutyrin was hormonally regulated, as the addition of epinephrine to adipocytes caused a 10-fold increase in the amount of monobutyrin secreted. These results indicate that monobutyrin synthesis is adipocyte-specific, occurs through an apparently novel particulate enzyme system, and is regulated in a hormone-dependent manner. The implications of these results for adipose physiology and angiogenesis are discussed.

Introduction

Adipocytes play a key role in systemic energy balance, serving as the major depot for energy storage. The ability to store and recall metabolic energy in a biochemically useful form depends upon an intimate relationship between the adipose cells and the vasculature. Hence, the formation and function of new blood vessels is central to the development and physiology of adipose tissue. Adipocyte differentiation and angiogenesis are tightly coordinated during embryogenesis and neovascularization is also required for further development of adipose tissue (1). Activation of lipolysis also brings about local vascular changes, most notably the dilation of small blood vessels and increased blood flow (2). Our previous work has shown that 3T3-F442A preadipocytes undergoing differentiation secrete factors which are strongly angiogenic *in vivo* and stimulate motility and mitogenesis of vascular endothelial cells *in vitro* (3-5).

One of these angiogenic factors was identified as the novel lipid 1-butyryl-glycerol (monobutyrin)[1] (6). The purified synthetic compound has been shown to promote angiogenesis in the chick chorioallantoic membrane assay and motility of isolated endothelial cells *in vitro*. Metabolic labeling studies utilizing various radiochemical precursors such as [^{14}C]acetate have indicated that this compound is synthesized in a differentiation-dependent manner when adipocytes are compared to preadipocytes. Monobutyrin levels were estimated to increase 200-fold during the differentiation process.

These results suggested that monobutyrin is a key regulatory molecule in the development of adipose tissue vasculature (6).

Little is known about the cell and tissue distribution of monobutyrin biosynthesis. The use of radiochemical labeling to quantitatively estimate the concentration of a particular product is subject to many assumptions regarding pool size, rate of uptake, and rate of clearance which vary for each cell type. Furthermore, there is nothing known regarding the pathway of biosynthesis of this factor in adipocytes. To address these questions, we have developed a facile enzymatic assay to investigate the production of monobutyrin by a variety of cell types and have also examined the enzyme(s) involved in the biosynthesis of this lipid angiogenic factor in broken cell preparations. These studies reveal that monobutyrin is synthesized by a novel enzyme or enzyme system and the production of this lipid is regulated by both chronic and acute mechanisms in adipocytes.

Materials and Methods

Materials

Glycerol kinases were purchased from Sigma. Pure monobutyrin was distilled from practical grade monobutyrin as described previously (6). 1-butyryl-[^{3}H]glycerol ([^{3}H]monobutyrin) with a specific activity of 4.2 x 10^{4} cpm/nmole was a kind gift of Dr. Henry Lu (California Biotechnology). [γ-^{32}P]ATP, [^{14}C]butyryl-CoA, and [2-^{14}C]glycerol were purchased from New England Nuclear. *sn*-[^{14}C]glycerol-3-phosphate was synthesized from [^{14}C]glycerol as described (7). Channeled silica gel thin layer plates were purchased from VWR. Sep Pak C18 cartridges were purchased from Waters Associates.

Cell Culture

3T3-F442A cells were grown as described (8). Other cell lines were grown in DME plus 10% fetal calf serum unless otherwise indicated.

Purification of Monobutyrin from Conditioned Media

Sep Pak C18 cartridges were washed with 5 ml glass-distilled water, activated with 5 ml of 95% ethanol, and washed again with 6 ml of glass-distilled water. One to 10 ml of conditioned media from cells was applied through the column, the eluate collected and passed through the column again. The cartridge was washed with 2 ml glass-distilled water and monobutyrin was then eluted with 2 ml of 50%

ethanol. Aliquots were dried under nitrogen gas for assay as described below. The remaining eluate was subsequently stored at $-80^{o}C$.

Enzymatic Determination of Monobutyrin

The 50% ethanol eluate from the Sep Pak fractionation was dried down under nitrogen gas in washed 13 x 100 mm screw cap borosilicate tubes (Fisher). A reaction mixture consisting of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, 400 U/ml glycerol kinase and 5 mM [γ-^{32}P]ATP (specific activity 20-40 cpm/pmol) was added in a final volume of 50 µl. The reaction was vortexed and incubated at $25^{o}C$ overnight. 25 µl of the reaction was spotted onto channeled thin layer silica plates and allowed to air-dry completely. The plates were developed in n-butanol/glacial acetic acid/water (2:1:1). The plates were allowed to dry and exposed overnight to Kodak XAR film. Bands corresponding to phosphomonobutyrin were scraped and counted in scintillation fluid.

Preparation of Cell Extracts

Cells were washed three times in ice-cold PBS and scraped in PBS into 50 ml Falcon tubes. Cells were pelleted and resuspended in 5 volumes of homogenization buffer (0.25 M sucrose, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride) and homogenized on ice with 20-30 strokes of a tight-fitting Dounce homogenizer. Lysis was monitored by trypan blue staining to detect free nuclei. This suspension was centrifuged for 15 min at 15,000 g at $4^{o}C$. The resulting supernatant was used as the whole cell homogenate. The

homogenate was centrifuged at 400,000 g for 20 min at 4°C to obtain the cytosolic (supernatant) and particulate (pellet) fractions. The pellet was resuspended in 0.5 volumes of homogenization buffer with 5-10 strokes of Dounce homogenizer and spun at 15,000 x g for 15 min at 4°C. The resulting supernatant was used as the particulate fraction and stored at -80°C.

Assay of Monobutyrin Synthesis in Cell Extracts

Samples were assayed in 75 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$ in the presence of various concentrations of radioactive precursors at 37°C in a final reaction volume of 40 µl at the times indicated. The reactions were terminated by the addition of 5 µl perchloric acid and immediately spotted onto channeled silica plates and air dried for at least 30 min. Plates were developed in ethyl acetate:isooctane:glacial acetic acid (11:5:2) saturated with water. Plates were then air-dried and sprayed with En^3Hance (New England Nuclear) before exposure to Kodak XAR-5 film.

Chemical Analysis of Phosphomonobutyrin and Monobutyrin

1-butyryl-3-phospho-glycerol (phosphomonobutyrin) was isolated from bands visualized after autoradiography by scraping the silica and eluting with methanol. The resulting solution was then spotted on thin layer plates and developed in three solvent systems in which phosphomonobutyrin did not co-migrate with any standards used (glycerol-P, glycerol, monobutyrin, or butyrate), indicating that a compound distinct from glycerol or glycerol-P was being formed upon phosphorylation of monobutyrin by the glycerol kinase. In addition,

this compound was base hydrolyzed by adding 1 N NaOH for 2 hrs at room temperature and the resulting mixture was subjected to thin layer chromatography. After base hydrolysis, either the ^{32}P or ^{14}C form of phosphomonobutyrin comigrated with glycerol-P in three different solvent systems.

Monobutyrin synthesized by cell-free extracts and co-migrating with authentic monobutyrin in the standard thin layer system (ethyl acetate:isooctane:glacial acetic acid, see above) was eluted from silica with methanol. Further proof of identity was obtained by co-chromatography of this band with authentic monobutyrin on three alternate thin layer chromatography systems. In addition, base hydrolysis of this band yielded [^{14}C]butyrate or [^{14}C]glycerol when [^{14}C]butyryl-CoA or [^{14}C]glycerol-P were used as substrates, respectively. These results together indicate that the compound being formed *in vitro* is monobutyrin.

Other Assays

Pure monobutyrin was quantitated using the ester assay (9). ATP purity was monitored using chromatography on polyethylimine cellulose in 1 M LiCl/M formate. Glycerol was measured according to Bradley and Keslow (10). Proteins were determined according to Read and Northcote (11).

Results

An Enzymatic Assay for Monobutyrin

There is currently no method available for measuring absolute amounts of monobutyrin. To study monobutyrin biosynthesis by cells in culture and its presence in various biological fluids, a simple quantitative method for measuring this molecule was developed. Our rationale was to use the glycerol kinase enzyme (E.C. 2.7.1.30) to phosphorylate the 3-hydroxyl of monobutyrin (12,13) with [γ-^{32}P]ATP of a known specific activity and isolate the resulting [^{32}P]phosphomonobutyrin from the reaction. To facilitate the screening of the enzymes, [^{3}H]monobutyrin was used as a substrate to examine several commercially available glycerol kinases. Only one enzyme, that isolated from *Cellulomonas sp*, was able to phosphorylate monobutyrin. As shown in Figure 1, this phosphorylation, which causes a change in mobility of the tritiated compound on the thin layer system, was essentially quantitative for monobutyrin levels ranging from 100 to 1000 pmol in the assay. The kinase activity was linear with added enzyme to 400 units/ml, after which increasing enzyme concentrations were somewhat inhibitory. This reaction was dependent upon the addition of enzyme, magnesium and at least 0.1 mM ATP, although 5 mM appeared to be optimal.

Direct assays of conditioned media for monobutyrin were not possible due to the presence of large amounts of glycerol and/or inhibitors of the glycerol kinase. An initial purification step was

necessary to separate monobutyrin from these compounds. As shown in Figure 2, quantitative recovery (97.4 ± 0.4%, n=4) of labeled monobutyrin from cell culture media was obtained by fractionation over Sep Pak C18 cartridges. The recovery from Sep Pak was quantitative at all concentrations examined (0.1 μM to 10 μM).

Using cell culture media containing known amounts of added purified monobutyrin, the assay is linear up to 1000 pmol (the highest amount tested) in a 50 μl reaction volume (Figure 3). Monobutyrin levels of 25 pmol or less had a wide range of deviation and therefore were considered below the level of detection for this assay. The enzyme itself (Figure 3A, *DME only* lane) generated a band which comigrated with phosphomonobutyrin. Base hydrolysis and subsequent thin layer chromatography suggested that this band was in fact phosphomonobutyrin. This background was subtracted out when determining monobutyrin values.

Secretion of Monobutyrin by Different Cell Types

Several different cell lines were examined for the ability to produce monobutyrin. Of the lines examined, including several transformed and tumor cell lines (e.g., sarcoma 180 and fibrosarcoma)[2], only adipocytes (*AD CM*) made detectable amounts of monobutyrin (Figure 4). In addition to the 3T3-F442A line, adipocytes derived from 3T3-L1 cells also made detectable amounts of monobutyrin (not shown). Based upon a limit of detection of 25 pmol, we estimate that cell-conditioned media of non-adipose cells must contain a concentration of less than 10^{-8} M. The steady state concentration of

monobutyrin in 3T3-F442A adipocyte-conditioned media was calculated to be 1.3 ($\pm$ 0.1) x 10^{-6} M (n=4).

In Vitro Synthesis of Monobutyrin by Cell Extracts

To examine the mechanism of monobutyrin synthesis, broken cell preparations of adipocytes were examined for the ability to synthesize monobutyrin *in vitro*. Adipocyte particulate and cytosolic fractions were incubated in the presence of 40 µM butyryl-CoA and radiolabeled glycerol-P (75 µM) as described in the Methods section. Monobutyrin synthetic activity was evident and appeared to be membrane-bound as the particulate fraction of adipocytes contained all the detectable activity while the cytosolic fraction contained none (Figure 5, lanes 6 and 3). Monobutyrin synthesis was linear with time (up to 1 hour) and protein (up to 250 µg). Synthesis was optimal at a pH range between 6.5 to 7.5 and was completely dependent on added magnesium. Monobutyrin synthesis was completely dependent upon the addition of butyryl-CoA if glycerol-P was used as the labeled substrate. However, monobutyrin synthesis was not dependent on or stimulated by the addition of glycerol or glycerol-P if [^{14}C]butyryl-CoA was used as a substrate, suggesting that an endogenous glycerol backbone donor is present in the particulate fraction.

Although monobutyrin was detected only in adipocyte-conditioned media (see Figure 4), it was possible that part of the enzymatic machinery for monobutyrin synthesis was present in non-adipose cells. 3T3-F442A preadipocytes and NIH-3T3 fibroblast particulate fractions were examined for monobutyrin biosynthetic activity. As shown in

Figure 5 (lanes 4-6), the activity is seen only in membranes from adipocytes and not in the other cell types tested. In addition, particulate fractions made from adipocytes isolated from rat adipose tissue also demonstrated monobutyrin synthetic activity (not shown). Mixing experiments did not indicate the presence of an inhibitor in non-adipocyte membranes or whole cell homogenates using either [^{14}C]glycerol-P or [^{14}C]butyryl-CoA as substrates.

Acylation of glycerol-P by butyryl-CoA could conceivably be carried out by the microsomal glycerol-P acyltransferase, whose activity is known to greatly increase in differentiating 3T3-L1 (19,20) and 3T3-F442A (21) preadipocytes. Glycerol-P acyltransferase optimally uses chain lengths of C16 to C18 and has been reported to ineffectively utilize acyl-CoA's of chain length 10 or lower (22). To determine if this enzyme was responsible for this reaction, competition experiments using [^{14}C]butyryl-CoA with increasing chain length acyl-CoA's were performed. Incubation of the *in vitro* monobutyrin synthetic reaction with acyl-CoAs of lengths C2-C12 showed a decreasing ability to compete with [^{14}C]butyryl-CoA as chain length increased beyond C8 (Figure 6). Longer chain lengths were not used in the assay due to their strong amphipathic properties (23). In addition, butyryl-CoA failed to inhibit the microsomal glycerol-P acyltransferase activity from adipocyte particulate fractions when palmitoyl-CoA was used as the substrate (not shown). These data suggest that the formation of monobutyrin is unlikely to involve glycerol-P acyltransferase and appears to utilize a specific and possibly novel enzyme system.

Monobutyrin Biosynthesis is Hormonally Regulated

As shown above, a major chronic regulator of monobutyrin synthesis is cell differentiation. Because changes in vascular state are known to occur during the activation of lipolysis (2), we asked whether a known lipolytic agent could modulate monobutyrin production in fully differentiated cells. Adipocytes were serum deprived overnight, stimulated with 10^{-7} M epinephrine and the conditioned media was assayed for monobutyrin. As seen in Figure 7, the unstimulated level of monobutyrin increased up to 4 hrs. The concentration then remained constant at approximately 1.0 μM up to 16 hrs (the longest time point examined). However, upon stimulation of the cells with epinephrine, the initial rate of monobutyrin secretion increased about 8-fold compared to the unstimulated state. After eight hours, the steady state monobutyrin level was maintained at 8 x 10^{-6} M for the remainder of the time period examined. Quantitation of glycerol release into the media showed an approximate 10-fold increase by the epinephrine-stimulated cells over control in the first four hours, indicating the activation of lipolysis. Nearly identical results were observed with two other lipolytic agents, 10 nM prostaglandin E_1 (24,25) and 0.5 mM dibutyryl cAMP (26). Therefore, the synthesis of monobutyrin by adipocytes appears to be subject to hormonal regulation involving a cyclic AMP-linked pathway.

Discussion

Monobutyrin is a novel lipid with angiogenic activity that we identified previously from adipocyte-conditioned media (6). As such, the ability to study the regulation of this factor is greatly facilitated by a quantitative assay for monobutyrin in conditioned media and other biological fluids. We describe here a facile assay for measuring monobutyrin levels in cell-conditioned media. This procedure requires only a simple one-step purification from media and subsequent phosphorylation of the compound via glycerol kinase. By using this assay, we have demonstrated that monobutyrin is present in micromolar quantities at steady-state levels in adipocyte-conditioned media and is not detectable in the cell-conditioned media of the other cell lines examined. We estimate that if monobutyrin is secreted by these cells, it must be present at a concentration of less than 10^{-8} M in conditioned media.

While use of the glycerol kinase assay indicates that the secretion of monobutyrin is differentiation-dependent, it is conceivable that the synthesis of the factor was not. We have demonstrated that the whole cell homogenate and particulate fractions isolated from adipocytes are able to synthesize monobutyrin *in vitro*. This synthetic activity appears to be adipocyte-specific and can utilize glycerol-P, butyryl-CoA, and to a lesser extent, glycerol (not shown) as substrates. Use of this assay allowed us to establish that the activity has a preference for short chain fatty acyl-CoA's (see

Figure 6). This specificity ruled out the possibility that the activity was due to the previously characterized microsomal glycerol-P acyltransferase, whose activity is known to increase greater than 70-fold during 3T3 differentiation (20,21). Our data is consistent with previous work that has shown that this acyltransferase assayed from adipocytes failed to utilize acyl-CoA's of C10 chain length or shorter (22).

The simplest enzymatic mechanisms to explain the synthesis of monobutyrin is either the acylation of glycerol-P by butyryl-CoA and subsequent dephosphorylation to monobutyrin or the direct acylation of glycerol with butyryl-CoA to form monobutyrin. These mechanisms would be analogous to the pathway of diacylglycerol synthesis (27) and consistent with the fact that radiolabeled glycerol-P and glycerol can be incorporated into monobutyrin. However, we have found that [^{14}C]butyryl-CoA can be used as a substrate by washed adipocyte particulate fractions without any other added substrates. Furthermore, adding glycerol or glycerol-P to these washed membrane fractions with [^{14}C]butyryl-CoA in the assay failed to increase the amount of monobutryrin produced. These preliminary results suggest that there must be an endogenous glycerol backbone generated from a compound present in the washed particulate fractions. The origin of the glycerol moiety utilized in monobutyrin synthesis is currently under examination.

In light of its angiogenic activity, the cell-type specificity of the monobutyrin molecule is somewhat surprising. Conditioned media

from several cell lines representative of actively metabolizing tissue that are vascularized (tumor, muscle, and embryonic) showed that none contained detectable amounts of monobutyrin. This suggests that the monobutyrin molecule may play a specialized role in adipocyte physiology. While the vascularization of adipose tissue could certainly utilize mechanisms distinct from other developing cell types, it is also possible that monobutyrin is a pleiotropic effector having multiple functions related to blood vessel biology, some specific for adipose cells. In this regard, the rate of blood flow and state of vasodilation in adipose tissue are both modulated in various metabolic states (2, 28). It is believed that increased blood flow facilitates the removal of fatty acids from adipose tissue during the fasting, lipolytic state (29). Interestingly, we show here that monobutyrin is acutely regulated by lipolytic factors.

Because monobutyrin production increases sharply at the onset of lipolysis, monobutyrin could be involved in some aspect of vasodilation and/or vascular permeability. The notion of a single molecule having both angiogenic activity and other biological actions is now well established. Fibroblast growth factor, tumor necrosis factor, and prostaglandins (just to name a few) have angiogenesis as only one of several biological activities (30-33). Similarly, vascular endothelial growth factor elicits many different responses even within the blood vessel systems, having been isolated independently as both an angiogenic factor (34) and a vascular permeability factor (35). Since angiogenesis appears to be a rather permissive biological response to many pharmacological effectors, the

cell type specifity and acute hormonal control over monobutyrin synthesis suggests that this molecule may have other, additional biological and physiological effects on vascular cells or on other cell types. These are currently under investigation.

Footnotes

1. Abbreviations used: glycerol-P, *sn*-glycerol-3-phosphate; phosphomonobutyrin, 1-butyryl-3-phospho-glycerol; monobutyrin, 1-butyryl-glycerol; DME, Dulbecco's Modified Eagle medium; PBS, phosphate buffered saline.

2. Several other cell lines, in addition to the ones shown in Figure 4, were examined for the presence of monobutyrin in their conditioned media. These lines are: C_2C_{12} mouse myoblast cell line, both the myoblast and myotube cell types (14); CH310T1/2 CLB fibroblast cell line (15); 3T3-L1 preadipocyte cell line (16); βTC3 pancreatic β-insulinoma cell line (17); HL60 promyelocytic leukemia cell line (18). all the above mentioned cell types and lines did not have detectable amounts of monobutyrin in their cell-conditioned media.

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Figure Legends

Figure 1. Quantitative phosphorylation of [^{3}H]monobutyrin by glycerol kinase. Increasing amounts of [^{3}H]monobutyrin (100-1000 pmoles, as indicated in each lane) were incubated with 10 units of glycerol kinase from *Cellulomonas* and run on silica gel thin layer plates as described in the Methods section. Arrows indicate where authentic monobutyrin and phosphomonobutyrin migrate. The *no enzyme* lane is 1000 pmoles of [^{3}H]monobutyrin added to the reaction mixture with no enzyme added. The band seen comigrating with phosphomonobutyrin in the *no enzyme* lane is contaminating [^{3}H]glycerol from the [^{3}H]monobutyrin preparation. The identity of phosphomonobutyrin was verified as described in the Methods section.

Figure 2. Quantitative recovery of monobutyrin from Sep Pak C18. DME plus 10% fetal bovine serum was spiked with 1.0 μM [^{3}H]monobutyrin. 2 ml of this mixture was put through a Sep Pak C18 cartridge as described in the Methods section. Equal volumes of each step of the fractionation were dried under nitrogen gas and resuspended in a minimal volume of water. The whole sample was spotted on thin layer plates and developed as described. Lane 1, starting media spiked with [^{3}H]monobutyrin. Lane 2, flow through. Lane 3, water wash. Lane 4, 50% ethanol eluate.

Figure 3. Titration of monobutyrin in the glycerol kinase-based assay. A. Autoradiogram of thin layer plate after glycerol kinase

reactions. Purified monobutyrin was added to DME plus 10% fetal calf serum and fractionated over Sep Pak C18. Assays of various amounts of the monobutyrin-bearing fraction (20 pmoles to 1.0 nmole, as shown in each lane) were performed as described in the Methods section. *DME only* refers to DME plus 10% fetal bovine serum without added monobutyrin. B. Quantitation of the glycerol-kinase based assay. An experiment similar to the one shown in A was performed. The regions corresponding to phosphomonobutyrin were scraped and counted. The resulting counts per minute were plotted versus the amount of monobutyrin added to the assay. The R coefficient for this line was 0.9967. The specific activity of the ATP used was 19.7 cpm/pmol.

Figure 4. Monobutyrin is specific to adipocyte-conditioned media. Media conditioned for 24-48 hrs by several different cell lines at confluence was analyzed for the presence of monobutyrin as described in Methods. The numbers shown correspond to conditioned media volumes (μl) after correcting for fractionation on the Sep Pak cartridge. Lanes: *DME only*, DME plus 10% serum; *AD CM*, adipocyte-conditioned media; *PREAD CM*, preadipocyte-conditioned media; *NIH3T3 CM*, NIH 3T3 (36) fibroblast-conditioned media; *S180 CM*, sarcoma 180 (37) cell-conditioned media (36); *BPV1 CM*, fibrosarcoma (38) cell-conditioned media; *REF CM*, rat embryo fibroblast (39) cell-conditioned media; *50 pmol MB*, DME plus 10% fetal bovine serum containing 50 pmoles monobutyrin added.

Figure 5. Synthesis of monobutyrin by cell extracts. Cytosol and particulate fractions of adipocytes, preadipocytes, and NIH3T3 cells

were prepared and assayed for monobutyrin production for 30 min at 37°C using 75 μM [^{14}C]glycerol-P as described in the Methods section. Lane 1, 60 μg NIH3T3 cytosol fraction; Lane 2, 60 μg preadipocyte cytosol fraction; Lane 3, 60 μg adipocyte cytosol fraction; Lane 4, 40 μg NIH3T3 particulate fraction; Lane 5, 40 μg preadipocyte particulate fraction; Lane 6, 40 μg adipocyte particulate fraction. Arrow indicates the position where authentic [^{3}H]monobutyrin migrates. Essentially identical results were obtained using 40 μM [^{14}C]butyryl-CoA for 8 min at 37°C.

Figure 6. Acyl-CoA competition for monobutyrin synthesis. 40 μM [^{14}C]butyryl-CoA and different concentrations of various length acyl-CoAs were added to 40 μg of adipocyte particulate fraction. The reaction was incubated for 8 min at 37°C and analyzed for monobutyrin synthesis as described in Methods. o-o , 40 μM competing acyl-CoA. ♦-♦ , 80 μM competing acyl-CoA. □-□ , 160 μM competing acyl-CoA.

Figure 7. Epinephrine induction of monobutyrin synthesis. Adipocytes were washed in DME plus 0.25% bovine serum albumin and incubated overnight in the same media. The cells were then treated with the same media with and without 10^{-7} M epinephrine added and aliquots of the media were taken at the various times indicated and assayed for monobutyrin. o-o , 10^{-7} M epinephrine added. □-□ , no addition.

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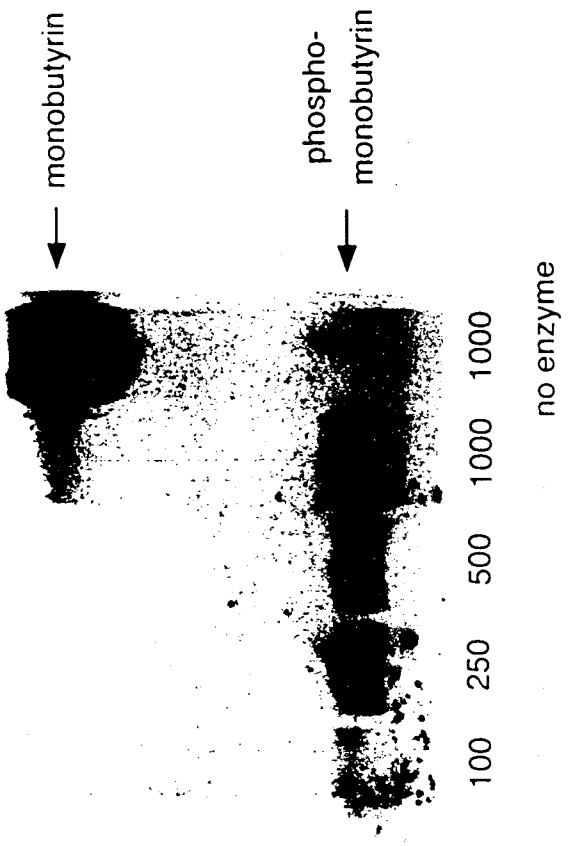

monobutyrin
phospho-
monobutyrin
100
250
500
1000
1000
no enzyme

FIG. 1

1	2	3	4
media alone	flow through	water wash	50% etoh eluate

FIG. 2

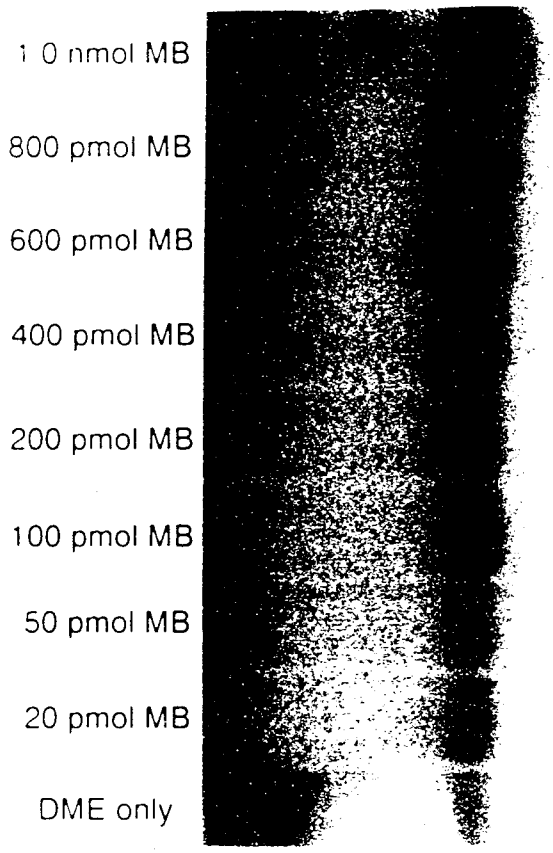

1 0 nmol MB
800 pmol MB
600 pmol MB
400 pmol MB
200 pmol MB
100 pmol MB
50 pmol MB
20 pmol MB
DME only

FIG 3A

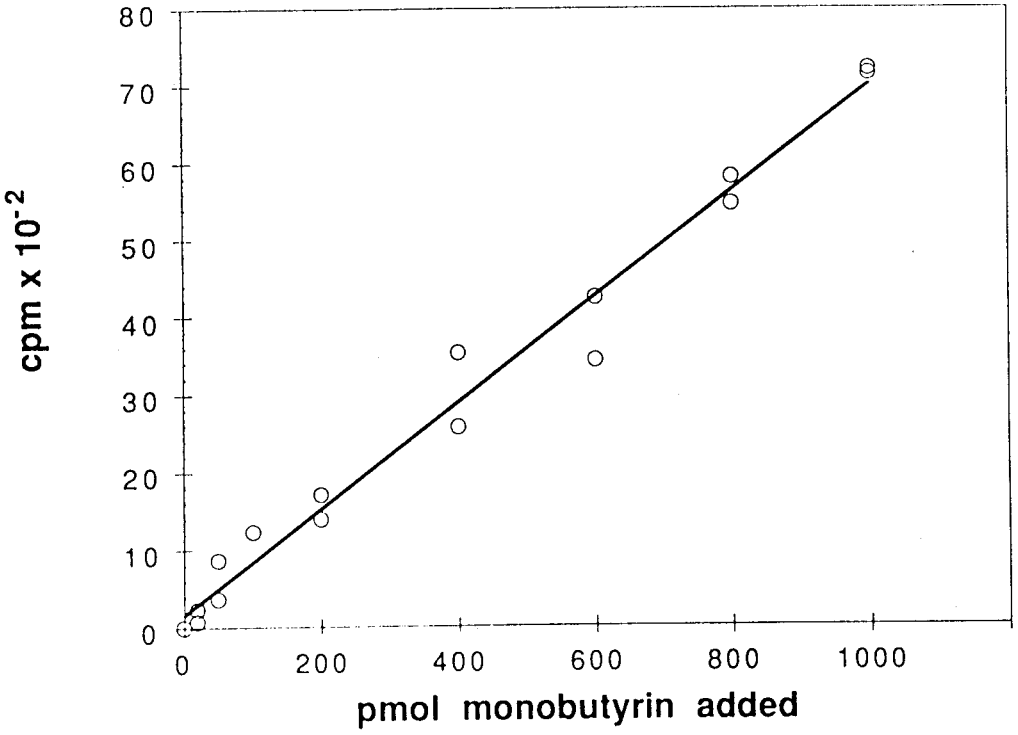

80
70
60
50
40
30
20
10
0
cpm x 10-2
0
200
400
600
800
1000
pmol monobutyrin added

Fig. 3B

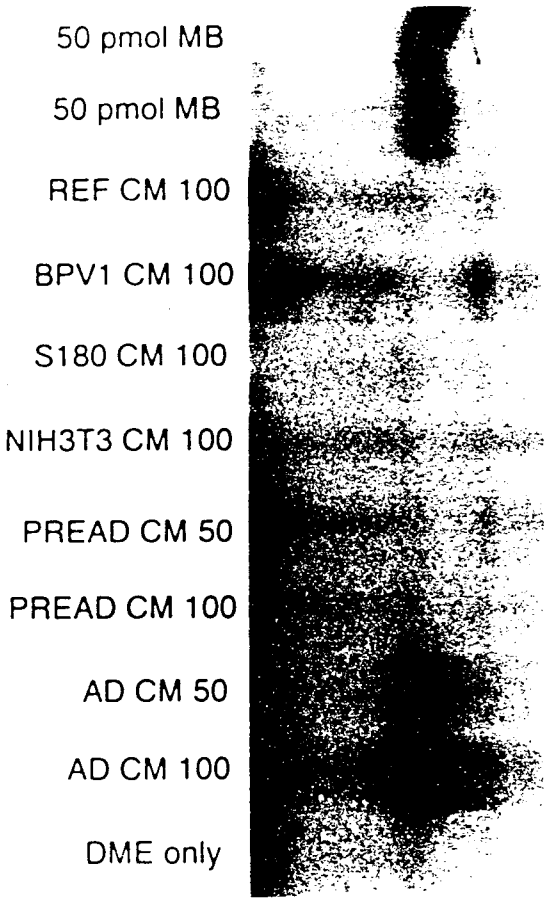
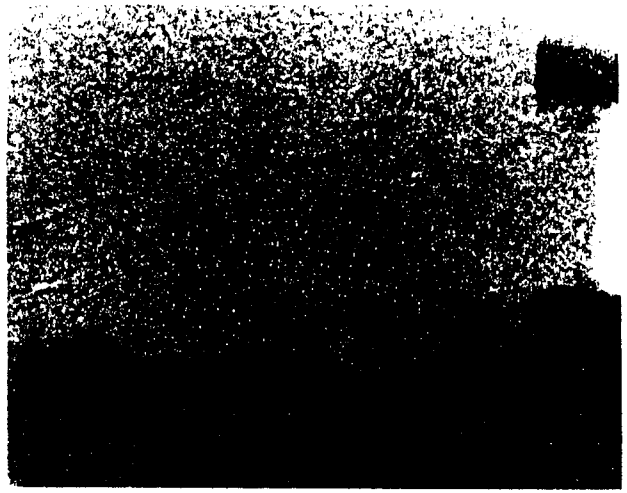

50 pmol MB
50 pmol MB
REF CM 100
BPV1 CM 100
S180 CM 100
NIH3T3 CM 100
PREAD CM 50
PREAD CM 100
AD CM 50
AD CM 100
DME only

FIG. 4

FIG. 5



1	2	3	4	5	6
NIH3T3 cyto	Pread cyto	Ad cyto	NIH3T3 part	Pread part	Ad part

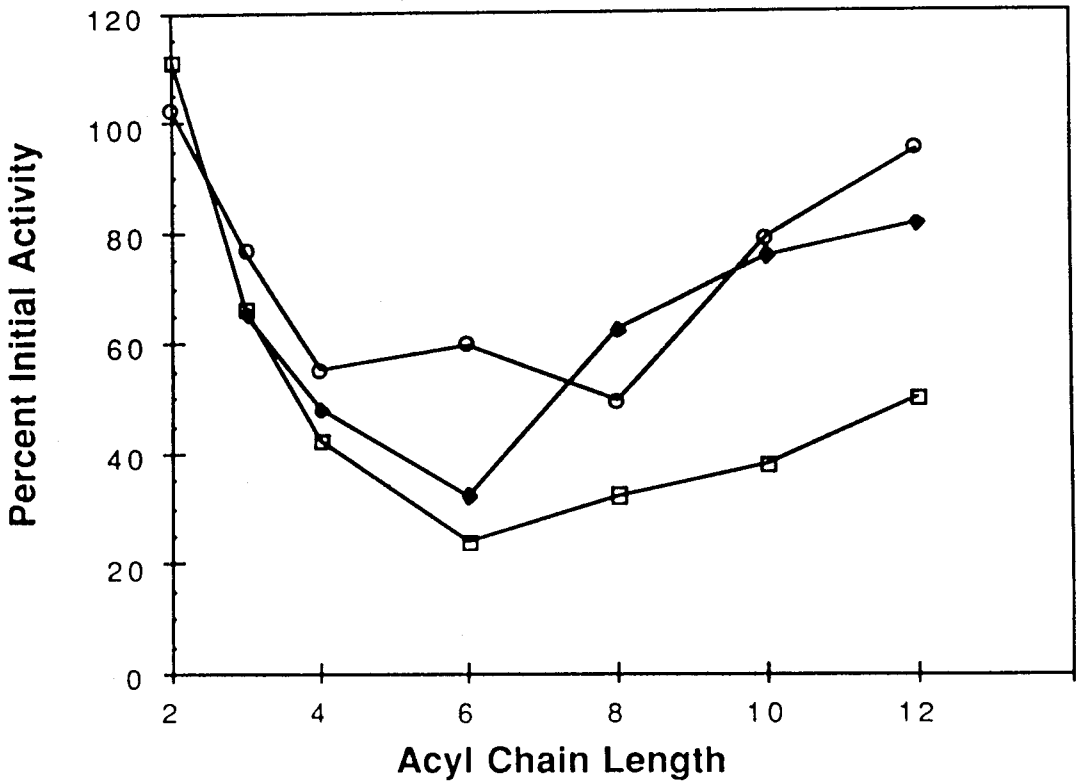

Percent Initial Activity
120
100
80
60
40
20
0
2
4
6
8
10
12
Acyl Chain Length

FIG. 6

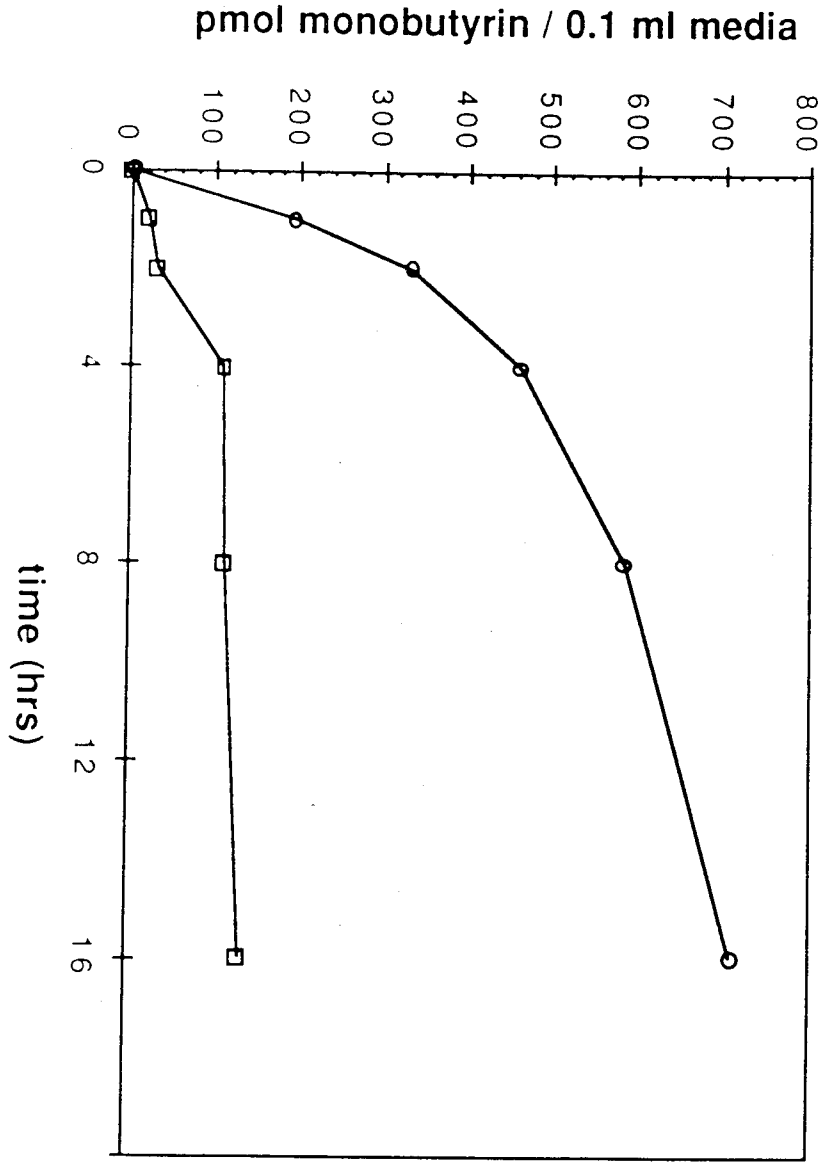

pmol monobutyrin / 0.1 ml media
0
100
200
300
400
500
600
700
800
time (hrs)
0
4
8
12
16

Fig. 7