AN ABSTRACT OF THE THESIS OF

Chu-Liang Chen for the degree of Master of Science in Animal Sciences presented on February 24, 1992

Title: Identification of an Inguinal Adipocyte-Specific Protein Using Monoclonal Antibody Selected by Cyclophosphamide

Signature redacted for privacy.

Abstract approved: _____

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Evidence suggests that adipose tissue is not a homogeneous entity, but rather that it differs functionally and morphologically according to its anatomical location. Production of a site-specific monoclonal antibody (MAb) is therefore of interest, not only for the study of adipocyte development and differentiation, but also for the elimination of adipocyte tissue at a specific site in vivo. Three adipocyte plasma membrane (APM) preparations were isolated from inguinal, epididymal, and perirenal fat depots of Sprague-Dawley rats. Two immunization approaches were applied in this study. In the original approach, BALB/c mice were immunized with inguinal APM proteins. An enzyme-linked immunosorbent assay (ELISA) method was developed to screen the hybridoma producing MAb specific to inguinal APM. The results of the screening procedure showed that all positive clones appeared to react with both epididymal and perirenal APM as strongly as with inguinal APM. In the alternative approach, BALB/c mice were immunized with a mixture of epididymal and perirenal preparations. The immunization was followed by the injection of cyclophosphamide to eliminate the antigen-stimulated

lymphocytes selectively. Following a three-week rest, the mice were immunized with the inguinal APM preparation. The same ELISA method as in the first approach was used to screen the hybridoma clones. Positive clones producing inguinal-specific antibody were selected. A unique protein with molecular weight estimated at 25 KDa was identified only in the inguinal plasma membrane preparation by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot techniques. Results from indirect-immunofluorescence demonstrated that this antigen is located on the plasma membrane of inguinal fat cells.

Identification of an Inguinal Adipocyte-Specific Protein Using Monoclonal Antibody Selected by Cyclophosphamide

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed February 24, 1992

Commencement June, 1992

APPROVED:

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Date thesis is presented February 24, 1992

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ACKNOWLEDGEMENT

I want to express my gratitude to Dr. Ching Yuan Hu, Dr. Gary M. Banowetz, Dr. Robert R. Becker, and Mrs. Nancy Wehr for their guidance, advice, help and support. Also, I wish to thank my parents and Peiyu, my fiancee, for their continued love and support.

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INTRODUCTION

Potential regional variation in adipose tissue growth among various fat depots is an intriguing perspective of the biology of this tissue and has long attracted interest. Adipose tissue samples from different sites display disparities in various aspects, such as cell number and size, rate of lipolysis, lipogenic enzyme activity, and expression of adrenoreceptor. Regional adiposity can be altered by sex, age, dietary intake, hormonal factors, and cigarette smoking. Furthermore, regional fat distribution has been shown to be associated with metabolic abnormalities and disease states. Abdominal obesity is strongly associated with risk factors for cardiovascular disease, cerebrovascular disease and non-insulin-dependent diabetes. However, enlargement of peripheral adipose tissue is not associated to the same degree with these conditions. Growth of adipose tissue is dependent not only upon deposition of lipid in existing mature fat cells, but also upon the production and filling of new fat cells. For the last decade, primary culture of fat cell precursors has been increasingly used to study the replication and differentiation of adipocyte precursors. Adipocyte precursor cells isolated from different locations have been shown to differ in their potential for hyperplastic growth. This suggests that the adipose tissue is not a homogeneous entity, but rather that it differs functionally and morphologically according to its anatomical location. Therefore, it is of interest to identify possible unique proteins in the adipocytes at a specific site, and subsequently to analyze the biochemical function and structure of

the identified protein(s) to delineate mechanisms leading to development of the specific adipose tissue.

Cell plasma membranes play a major role in intercellular communication, nutrient transport, and recognition of hormone messages. Immunological reagents have been produced and used to study some antigenic components present in the adipocyte plasma membrane. The major advantage of this immunological approach is that the antibody distinguishes a particular cell type among morphologically similar cells. Polyclonal sera developed against adipocytes are able to identify species-specific compositional differences in adipocyte plasma membrane (APM). In a recent study, a monoclonal antibody (MAb) directed against porcine adipocytes identified a unique APM protein which is expressed only on adipocytes from genetically lean but not obese pigs. MAbs, specific to adipose tissues from either swine or rats, have also been produced by two other groups to study adipose tissue development. Although regional differences in adipose tissue indicate indirectly the existence of site specific proteins on the surface of cytoplasmic membranes, adipose tissues in different sites are still quite similar biochemically and share major plasma membrane components. This suggests that the site specific proteins may be a non-dominant structural component on the adipocyte surface. The normal immune response shows a considerable bias toward producing antibodies to a few immunodominant determinants. Therefore, antibodies against minor, site specific APM constituents are more difficult to produce. Cyclophosphamide, an immunosuppressive drug, has been used to manipulate the direction of the normal immune response. Appropriate application of cyclophosphamide with immunogens can eliminate actively dividing clones of lymphocyte producing antibodies against

immunodominant, non-site specific proteins and enhance the probability of producing antibodies to the non-dominant site specific cell surface proteins.

This thesis describes the production of MAbs by two approaches. The first used a traditional immunization protocol with rat inguinal APM as antigen. MAbs produced not only reacted with inguinal APM, but also cross-reacted with the APM proteins from the epididymal and perirenal sites. In the second approach, the application of cyclophosphamide enabled production of a MAb which recognized a unique protein present only in rat inguinal APM. This inguinal-specific protein was identified and its molecular weight estimated to be 25 KDa using the western blot analysis.

LITERATURE REVIEW

1. Regional variation of adipose tissue metabolism among various fat depots

The main function of adipose tissues is to store triglycerides during periods of affluence and to release the stored lipids as fatty acids and glycerol when the energy is needed. Tools of modern biochemistry and cell biology have allowed extensive studies and understanding of the manner in which ingested calories are stored and released from adipose tissue. However, potential regional variation in adipose tissue growth among various fat depots is an intriguing perspective of the biology of this tissue and has long attracted interest in both animal and human studies. Adipose tissue samples from different sites display disparities in various aspects, such as cell number and size, rate of lipolysis, lipogenic enzyme activity, and expression of adrenoreceptor. In this section, discussion will focus on the experiments and results in the literature according to the animal model used.

Rats

Benjamin et al. (1961) found that there were significant differences in the metabolic activity, based on incorporation of [^{14}C] acetate into mixed lipids of adipose tissue samples from various anatomical locations in rats. When the adipocytes were isolated from different anatomical locations in rats, differences in metabolic activity were observed (Fried et al., 1982). In addition, differing levels of hormone receptors in rats have been suggested

based on the observation that adipocytes from different sites tend to have varying affinities to hormones (LaFranchi et al., 1985).

Pigs

Adipose tissues from areas where fat is deposited very readily, particularly the perirenal region, had higher enzyme activities, larger adipocytes, a lesser amount of stromal tissue, a greater amount of ether extractable lipid, and a lower concentration of adipocytes per gram of tissue than samples from areas where fat is deposited only sparsely (leg subcutaneous) in male castrate pigs (Anderson et al., 1972). Growing pigs were nutritionally manipulated to study depletion, depletion followed by repletion, or deposition of the individual backfat layers. This study indicated that backfat was neither deposited nor utilized to the same extent at all anatomical locations and that outer layer was considerably more stable than second layer during both depletion and deposition or repletion of backfat in pigs (Mersmann and Leymaster, 1984).

Humans

In men, lipoprotein lipase (LPL) enzyme activity was higher in abdominal than in gluteal adipose tissue, whereas the opposite was observed in women (Arner et al., 1991; Raison et al., 1988). Additionally, there are regional variations in the steady state expression of lipoprotein lipase in human subcutaneous adipose tissue. Women have more LPL in the gluteal and femoral adipose tissues, where fat cells are bigger, than they do in the abdominal adipose tissue (Rebuffe-Scrive et al., 1985). High LPL and α_2-adrenergic receptor activities may tend to favor the deposition of lipid in this anatomical region (Rebuffe-Scrive et al., 1985).

Lipolytic response to norepinephrene, which activates both β_1- and α_2- receptors, is more prominent in abdominal than gluteal or femoral tissues in both male and female subjects. Furthermore, high level of α_2-receptor (anti-lipolytic) activity in gluteal depots and a preponderance of β_1-receptor (lipolytic) activity in abdominal sites of both men and women were observed (Leibel and Hirsch, 1987).

The abdominal depots are more responsive and sensitive to lipolytic hormones than the peripheral depots. Therefore, triglycerides in the abdominal region have a more rapid turn-over *in vivo*, and, as compared with a similar expansion of the peripheral depots, an expansion of the abdominal depots would lead to enhanced fatty acid release to the liver (Marin et al., 1987).

Evaluation of quantitative and/or qualitative differences among adipocyte plasma membranes (APMs) from specific sites suggested that adipocyte site specific proteins may exist (Nassar and Hu, 1991b). Immunoblotting using polyclonal antibodies raised against subcutaneous APM proteins demonstrated that several bands were missing from perirenal APM compared to the subcutaneous APM.

These observations of functional differences alude to the hypothesis that compositional differences exist in APM from different anatomical locations.

2. Effect of physiological factors on the alteration of regional adiposity

Adipose tissue at different locations responds dissimilarly to some physiological factors. These include gender, age, hormonal factors, dietary intake, and cigarette smoking.

Gender

"Android" or preponderant abdominal fatness, usually associated with maleness, is a specific risk factor for hypertension and diabetes. In contrast, femoral-gluteal or "gynecoid" obesity conveys either a greatly diminished or no risk for these disorders (Hirsch et al., 1989). LPL activity, which largely parallels variations in fat cell size, exhibits significant gender and regional differences. Premenopausal women have higher LPL activities in the gluteal and femoral regions than men (Fried and Kral, 1987), and these differences disappear after menopause (Rebuffe-Scrive et al., 1986). Furthermore, the proportion of body fat is higher in non-obese women than in non-obese men.

Arner et al. (1991) reported that in both abdominal and gluteal subcutaneous adipose tissues, LPL enzyme activity and mRNA levels were significantly higher in non-obese women than in non-obese men. Other studies indicated that in the majority of men, adiposity is largely confined to the upper parts of the body, such as back of the neck, shoulders, and abdomen. In women, however, the adipose mass may predominate in the upper body segment as in males or affect mainly the lower segment, such as buttocks and thighs.

Age

As described in the previous section the comparisons of the functional status of plasma membrane adrenergic receptors indicated that abdominal adipocytes are more responsive to the lipolytic action of β_1-adrenergic agonists, while gluteal adipocytes are more responsive to the antilipolytic action of α_2-adrenergic agonists in adult humans (Leibel and Hirsch, 1987). However, in children, there are no significant regional differences in either the basal rate of lipolysis or the responses to adrenergic lipolytic and anti-lipolytic stimuli. Furthermore, abdominal subcutaneous adipocytes from children had a significantly lower rate of basal lipolysis and were more responsive to α_2-adrenergic (anti-lipolytic) stimuli than abdominal adipocytes in adults. (Rosenbaum et al., 1991)

Schwartz et al. (1990) compared body composition, circumferences, and specific fat depot areas in a population of healthy young and older men. Although the two groups were similar in body mass index and percent body fat, the distribution of adiposity varied. The young subjects had larger thigh and arm circumferences, respectively, while the ratio of waist-to-hip circumference was greater in the older subjects. In addition, the older subjects had greater intra-abdominal fat area and lesser thigh subcutaneous fat area. They concluded that an age-related central and intra-abdominal re-distribution of adipose mass is present, even in the healthy older subjects.

Hormonal factors

Given the apparently equal degree of β_1 responsiveness in abdominal tissue of both sexes, the norepinephrine data suggested that men have more abdominal α_2 receptor function (anti-lipolysis) than women (Leibel and Hirsch, 1987). It implies that women have less of this anti-lipolytic activity than men in the abdominal region.

During pregnancy an increase in adipose tissue lipid stores occurs in women. The femoral region in women has metabolic characteristics (eg. high LPL, low lipolytic response to norepinephrine) specifically favouring fat accumulation that makes this region the most likely site for lipid storage during pregnancy (Naismith, 1969).

A clear anti-lipolytic effect of insulin has been demonstrated in abdominal and gluteal adipocytes but not in the femoral adipocytes. This complete lack of anti-lipolytic effect was also previously reported in adipocytes from the distal femoral region (Smith et al., 1979). The stimulatory effect of norepinephrine on lipolysis was significantly greater in the abdominal than in the femoral region in women (Lindberg et al., 1991). Furthermore, lipolysis in subcutaneous abdominal adipocytes is more responsive to catecholamine stimulation than lipolysis in femoral subcutaneous adipocytes (Kather et al., 1977; Lafontan et al., 1979).

Dietary intake

Lipogenesis activity in adipose tissue is highly dependent on diet and adipose tissue location. When a group of animals fed a sunflower seed oil diet (14.7%) was compared to those with controlled low fat diet, pronounced anti-lipolytic action of insulin was observed only in subcutaneous adipocytes, not in the perirenal adipocytes (Benmansour et al., 1991). Also, insulin response was higher with the sunflower seed oil diet and lower with the controlled low fat diet only in the subcutaneous site (Field et al., 1988). Nonetheless, the perirenal site had a higher lipogenic activity than subcutaneous site in both the sunflower and the controlled diet.

Cigarette smoking

Cigarette smoking has also been correlated with adipose distribution. In studies of males, a dose-response effect between waist/hip ratio and number of cigarettes smoked is present. Although smokers weighed significantly less than non-smokers, they have significantly higher waist-to-hip ratio, adjusted for age and body mass index, than non-smokers (Shimokawa et al., 1989; Troisi et al., 1991).

3. Association of regional fat distribution with metabolic abnormalities and disease states.

Regional fat distribution has been shown to be associated with metabolic abnormalities and disease states beyond what is explainable by general adiposity. The relationship between anatomical distribution of adipose tissue and associated morbidities, such as diabetes, hypertension, hypertriglyceridemia, and atherosclerosis, was first emphasized by Vague (1956). Abdominal obesity has been found to be associated with specific metabolic aberrations and diseases in a number of cross-sectional and longitudinal studies (Lapidus et al., 1984). Only the male type of fatness (android obesity) is associated with metabolic and cardiovascular complications to obesity. This association is observed even in moderate forms of android obesity. The female type of obesity (gynecoid fatness) has no such association and becomes dangerous only in grossly obese subjects (Kissebah et al., 1982; Bjorntorp, 1985a).

Abdominal adipose tissues are comprised of subcutaneous abdominal fat and intra-abdominal fat that differ in cell size, metabolic activity, and drainage to circulation. Distribution of excessive fat on the abdomen, as measured by the waist to hip girth ratio (WHR), is associated with reduced glucose tolerance, insulin resistance, and the risk for diabetes. Intra-abdominal fat appears to play a more important role in decreasing insulin sensitivity than subcutaneous abdominal fat. Increase in intra-abdominal fat which is independent of the influence of BMI (body mass index, the weight in kilograms over height in meters, squared (BMI = $(kg/m)^2$)) has been suspected to result in decreases in insulin sensitivity even in healthy young men (Park et al., 1991). Although the explanations

for the association between intra-abdominal fat and decreased insulin sensitivity were not complete, intra-abdominal adipocytes are recognized to be smaller but metabolically more active than adipocytes located in subcutaneous abdominal depots (Rebuffe-Scrive et al., 1989).

More importantly, there is now considerable evidence to support the view that enlargement of central, abdominal adipose tissues is closely associated with disease, while enlargement of peripheral adipose tissue does not show such associations to the same degree. Specifically, cardiovascular disease, cerebrovascular disease, and non-insulin-dependent diabetes (NIDDM) are all closely associated with abdominal obesity. Gluteal-femoral obesity is also likely to carry an increased risk of NIDDM development, but less-marked than in the case of abdominal obesity (Bjorntorp, 1990). Apart from this, only varicose veins and joint problems have been found to be positively related to peripheral or, more precisely, gluteal-femoral obesity. The association between obesity and cardiovascular disease is found only in the abdominally obese population by Bjorntorp (1985a). Distribution of adiposity as assessed by waist/hip ratio is significantly correlated with coronary atherosclerosis in both females and males (Thompson et al., 1990).

More recent reports have focused on adipose tissue distribution and metabolism as a potential pathogenetic factors, or on a possible primary endocrine profile, which secondarily produces a specific adipose tissue distribution and function, leading in turn to complications associated with obesity (Kissebah et al., 1982; Krotkiewski et al., 1983).

4. Variation of preadipocyte growth potentials in different locations

Growth of adipose tissue is dependant not only upon deposition of lipid in existing mature fat cells but also upon the production and filling of new fat cells. There is broad agreement that in all species studied adipocyte size is changed more readily than fat cell number. Actually, nearly all obese subjects at any level of obesity have fat cell enlargement or hypertrophy. Nonetheless, extreme degrees of obesity are plausible to be accompanied by hyperplasia (Hirsch et al., 1989), since cells cannot enlarge unlimitedly and increase in cell number (hyperplasia) can be induced. Adipocyte hyperplasia is far more difficult to evaluate because the methods used are dependent on accurate estimates of total body fat as well as accurate sampling from representative depots for the determination of an average adipocyte size. Recently, based on the recognition that the white adipose tissue of both young and mature animals including humans can be expanded by cellular hyperplasia in addition to hypertrophy, intense interest on the nature, origin, proliferation and differentiation of adipocyte precursor cells has increased (Cryer et al., 1984).

For the last decade, primary culture of fat cell precursors has been increasingly used to study the replication and differentiation of adipocyte precursors. The isolation of cells from the white adipose tissue of animals and humans that are capable of differentiation into adipocytes *in vitro* and reimplantation *in vivo* has helped stimulate the formation of new concepts for the cellular mechanisms of regulation during adipose tissue development (Cryer et al., 1984).

Differences in potential for adipose tissue growth among various fat depots and among individuals have long attracted interest, but their basis is

still largely unknown. Cell culture techniques conducive to replication and differentiation of adipocyte precursors isolated from mature adipose tissues have been used mostly for the elucidation of mechanisms. Studies have been carried out primarily in rats and humans.

Rats

Rat precursors of perirenal origin replicate more rapidly than precursors derived from epididymal depots. Precursor populations from both depots are polyclonal with respect to replicative capacity, and the perirenal population contains a greater proportion of rapidly replicating clones. Perirenal precursor populations display more extensive differentiation than epididymal cell pools (Djian et al., 1983, 1985). From these data, it has been speculated that sites affect the frequency of clones with a high capacity for replication and full differentiation, and the relationship between capacities for replication and differentiation (Kirkland et al., 1990).

Other evidence supporting regional differences in preadipocyte composition is that site-specific morphologic and growth differences in preadipocytes were observed in primary and secondary cultures of rat preadipocytes from epididymal and retroperitoneal fat pads. Preadipocytes from epididymal but not retroperitoneal depot were found to cluster in clumps. The finding of site-specific differences in preadipocyte differentiation at low but not high plating densities reinforces the theory of diverse cellular composition in different fat depots (Djian et al., 1983; Lau, 1990). These *in vitro* observations may suggest implications to regional fat distribution in man.

Turkenkopf et al. (1988) determined LPL activity, total lipogenesis, and rates of growth for stromal-vascular cells derived from epididymal and inguinal depots of genetically obese and lean Zucker rats and determined that in the inguinal depot, on day 4, there was no significant effect of genotype on LPL activity. However, by day 6 cells derived from obese rats had increased LPL levels compared to those derived from lean animals. By day 8 this effect was substantial under both basal and insulin-supplemented conditions. In the epididymal depot, cells derived from obese rats showed increased LPL activity compared to those derived from lean animals only at day 8, and this difference was significant only under basal conditions. No genotype effect on LPL activity in epididymal cells grown in insulin-free medium for 7 days was noticed. A closer examination of the effect of genotype in the epididymal- versus inguinal-derived cells revealed a more exaggerated regional difference between genotypes in epididymal-derived cells. For example, at a concentration of 400 mU insulin in the growth medium, the lipogenesis ratio of lean to obese was approximately 10:1 in contrast to 2:1 in cells derived from the inguinal depot (Turkenkopf et al., 1988).

Differentiation of fat cell precursors was assessed by the appearance of LPL and glycerol-3-phosphate dehydrogenase (GPDH). LPL activity was found at the same level in cells of both deposits while GPDH activity was elevated in inguinal versus epididymal derived stromavascular cells. The different adipose conversion pattern of each culture was confirmed by morphological quantification: the maturation of epididymal fat cell precursors was faster but less extensive. The stromavascular fraction of inguinal and epididymal fat pads of 4 week-old rats was studied by electron microscopy (Gregoire et al., 1990).

Various fat depots in rats have widely dissimilar degrees of hyperplastic growth. It is interesting that cultures of retroperitoneal cells invariably advanced to confluence with normal monolayer growth, whereas cultures of epididymal cells always formed numerous clumps with multiple cell layers. LPL activity was higher in retroperitoneal than in epididymal cultures (13.9 +- 2.4 vs 8.9 +- 3.1 units/mg protein, $P < 0.05$). GPDH activity in retroperitoneal cultures at confluence was significantly higher than in epididymal cultures (15.3 +- 2.3 vs 3.3 +- 0.1 units/mg protein, $p < 0.001$) (Sztalryd and Faust, 1990).

Humans

Regional variations were also found in human adipocyte precursor cell cultures. Roncari et al. (1981) demonstrated that precursor populations derived from omental adipose tissue of massively obese subjects (greater than 170% of ideal body weight) replicated much more rapidly than those from normal weight subjects.

Another experiment assessed the extent of adipose differentiation by determination of GPDH activity after 18 days in culture. This enzyme activity was significantly higher in cultured cells from the abdominal region compared to those from the femoral depot (253.1 +- 40.9 vs 155.8 +- 21.4 mU/mg protein, $P < 0.01$). Rate of adipose conversion in cultured stromalvascular cells from the subcutaneous abdominal tissue was higher than that in cells from the femoral depot (Hauner and Entenmann, 1991). This study supported the suggestion of regional differences in the capacity of adipose tissue depots to form new fat cells.

5. Use of an immunological approach in the study of adipose tissue: polyclonal antibody

Polyclonal sera have been prepared against adipocytes from mice (Plaas et al., 1981), rats (Pillion et al., 1979; Thompson and Abraham, 1979; Lee et al., 1986), cattle (Cryer et al., 1984), sheep (Nassar and Hu, 1991a), and chicken (Lee et al., 1986; Dong and Hu, 1991). These antibodies have been used to study adipocyte development and specific metabolic activities.

Adipose tissue development

Marker enzyme activities in adipocyte differentiation displayed by precursor cells isolated from mature adipose tissues and from embryonic tissues are now well characterized. The activities are compatible with the morphology of mature adipocytes following differentiation in culture. However, relatively little is known of cell surface changes that might accompany the process of differentiation or the character of the precursor cell prior to its differentiation in culture. It is possible to use cell surface changes to characterize adipocyte differentiation in precursors of mature tissue. Since immunological assay methods have proven very useful in the study of cell surfaces in many other differentiating cell systems (Williams et al., 1977; Trisler et al., 1979; Funkhouser and Peterson, 1989; Sidhu,1979), the possibilities for the use of an immunologically based technique for studying adipocyte differentiation have been pursued.

One approach for studying events regulating cell differentiation involves identification of developmentally regulated macromolecules, including cell surface antigens. Using SDS-PAGE, several researchers (Czech and Lynn, 1973; Kawai and Spiro, 1977; Tume et al., 1985) have

demonstrated the presence of species-specific differences in APM composition. Similarly, using immunological techniques with polyclonal antibodies developed against adipocytes, several laboratories have identified compositional differences in adipocyte plasma membrane of various species (Thompson and Abraham, 1979; Plaas et al., 1981; Cryer et al., 1984). The possible existence of adipocyte-specific plasma membrane proteins was also suggested in experiments using immunoblotting and immunoprecipitation with polyclonal antibodies that recognized species-specific adipocyte surface components (Tume et al., 1985; Lee et al., 1986).

Antisera against adipocyte plasma membranes have demonstrated the presence of cell surface antigens which increase quantitatively during adipocyte differentiation *in vitro* (Cryer et al., 1984; Cryer, 1985). Using species and cell specific anti-adipocyte antisera, an immunoprecipitation procedure was developed that allowed the nature of adipocyte cell surface antigens to be investigated. The demonstrable post-confluent increase in cellular immunoreactivity exhibited by white adipocyte precursors *in vitro*, was attributed to the development of specific cell surface antigens on the mature adipocyte (Lee et al., 1986).

Specific metabolic activities

Available immunological techniques also have provided powerful tools in efforts to characterize membrane transport components and probe the mechanism by which insulin activates adipocyte hexose transport activity. Antibodies against adipocyte intrinsic membrane proteins (glucose transport syatem) reacted with an antigenic site on the outer surface of intact fat cells and mimicked the action of insulin by stimulation of hexose uptake. These antibodies apparently did not interact with the insulin

binding site of the adipocyte insulin receptor. The data demonstrated that antibodies against rat adipocyte intrinsic membrane proteins bound to the outer surface of the rat adipocytes, stimulated glucose transport and inhibited norepinephrine-stimulated lipolysis (Pillion et al., 1979).

In another report, antiserum produced against mammary adipocytes distinguished these adipocytes from mammary epithelial cells and fibroblasts and was used to identify adipocytes. After a specific antigen of the mouse mammary adipocyte was defined, the role of the adipocyte in mammary gland function and the role of cell to cell interaction in mammary gland function was investigated (Thompson and Abraham, 1979).

6. Use of an immunological approach in the study of adipose tissue: monoclonal antibody

Monoclonal antibody technology greatly facilitating immuno-targeting was developed in the 1970s. One advantage of the technique is that, unlike conventional polyclonal antibodies, great specificity could be achieved without the use of biochemically pure immunogens. It was not necessary to specifically identify the antigen or to know the biological function of the antigenic molecule since antibody-producing cells could be cloned in culture after identification by a suitable assay (Funkhouser and Peterson, 1989).

Monoclonal antibodies to cell surface antigens have proven to be a very powerful for examining the surfaces of cells during differentiation. Readily identifiable cell specific antigens are found on many mature cells. Cells proximal in a cell lineage often show a changing pattern of antigenic surface molecules (differentiation antigens). The use of immunological techniques has significantly contributed to the understanding of the lineage of many cells and the molecular processes responsible for their differentiation. The approach has been successfully applied to a wide variety of cell types and organ systems. These include hematopoietic cells, neurons, muscle, early embryos, kidney, and most impressively, in terms of depth of the studies, to normal and malignant lymphocytes (Funkhouser and Peterson, 1989).

In recent studies, Killefer and Hu (1990a) developed monoclonal antibodies against porcine adipocytes and identified a unique porcine adipocyte plasma membrane protein; Wright and Hausman (1990a) also

produced monoclonal antibodies against cell surface antigens to study porcine adipocyte differentiation.

In genetically lean and obese rat models, dietary manipulation has very little effect on body composition. This suggests that genetic factors play an intricate role in predisposing individual animals to either leanness or obesity. A monoclonal antibody to an adipocyte-specific plasma membrane protein was used to examine the differential expression of this protein in genetically lean and genetically obese pigs (Killefer and Hu, 1990b). Identification of a 64 kDa protein present in genetically lean but not genetically obese APM indicates the presence of novel adipocyte-specific surface proteins associated either directly or secondarily to the onset of obesity.

A vast amount of information has accumulated demonstrating that changes in cellular morphology, as well as concomitant changes in cellular enzymatic activities associated with lipid accumulation, characterize adipocyte differentiation *in vivo* and *in vitro*. However, the exact identity of the cells contributing to the adipogenic cell subpopulation is unknown. Therefore, monoclonal antibodies were developed by Wright and Hausman (1990b) to identify specific developmentally-regulated cell surface components and to examine the expression of such components *in vivo* as well as *in vitro*. The identification of the cell surface antigens would be useful for marking adipocytes at some point prior to maturation. Two monoclonal antibodies were prepared by fusion of mouse myeloma cells and lymph node cells of mice immunized with a crude porcine APM fraction. These probes were used to study changes in cellular morphology in undifferentiated cells from presumptive adipose tissues cultured under conditions designed to either promote or suppress adipocyte differentiation

(Wright and Hausman, 1990a, 1990b). Cells along the adipogenic lineage possess cell surface antigens which may not be unique to adipogenic cells, but do exhibit differential expression among cell populations with adipose tissues. Furthermore, it has been suggested that the earliest cells to differentiate in culture are predetermined to some degree. By utilizing cell surface-reactive monoclonal antibodies, it may become possible to separate cells within developing adipose tissue into subpopulations for use in *in vitro* studies aimed at elucidating the developmental potential of the subpopulations.

7. Use of cyclophosphamide

When monoclonal antibodies are prepared using a mixture of proteins such as tissue extracts, there is a strong bias in favor of generating antibodies to a few immunodominant antigens. Antibodies against weak antigens are consequently more difficult to obtain. Therefore, methods that favor the production of antibodies to weak antigens would be useful. The immune system responds to any particular antigen in a complex manner which depends upon the characteristics of each antigen and the immunological history of the animal. This response involves the proliferation of distinct populations of lymphocytes. If mitotic cells are selectively killed following immunization, the immune system will contain a repertoire of lymphocytes capable of a different immune response following subsequent antigen challenge.

Cyclophosphamide is a potent suppressors of antibody formation. It has been used as an immunosuppressive drug in kidney transplants and severe rheumatoid arthritis, and prophylactically in patients undergoing bone marrow transplants. Cyclophosphamide has also been used to tolerize mice to one set of antigens followed by immunization with a similar but slightly different set of antigens. This approach yields an enhanced frequency of antibodies that distinguish the two sets of antigens (Matthew and Sandrock, 1987).

Cyclophosphamide is a fine, white, and almost odorless crystalline powder. It liquifies upon loss of its water of crystallization and discolors on exposure to light. It is soluble in water and in alcohol, and is slightly soluble in ether. It has been observed that rapidly dividing cells are more susceptible to the action of cyclophosphamide, a cytotoxic drug, than non-

cycling cells (Turk and Poulter, 1972). Cyclophosphamide reacts chemically with DNA. It kills cells during all phases of the cell cycle; that is, it is cell-cycle nonspecific, but it is more effective in the late G1 or S phases when the structures of nucleic acids are changed or unpaired. The principal target of cyclophosphamide has been speculated to be the 7-nitrogen of guanine in DNA that cross-links the two strands. This action prevents unwinding, causes deguanylation and base-mispairing, and compromises the template function of DNA. Because these processes are more prevalent in rapidly dividing cells, the use of cyclophosphamide leads preferentially to functional damage in rapidly proliferating cells. It has been shown that cyclophosphamide can affect a variety of lymphoid and non-lymphoid cell populations (Bach and Strom, 1985). Antibody formation to T-dependent as well as to T-independent antigens is suppressed by cyclophosphamide (Sabbele et al., 1988). Cyclophosphamide was therefore chosen to manipulate the immune response during the immunization for the production of adipocyte site-specific monoclonal antibodies in this study.

8. Potential of immunological approaches for increasing animal production

Current meat animal production systems produce a large amount of fat in carcasses. About two thirds of this fat is essential for biological functions and for meat palatability while the remaining one third represents an unneeded commodity. Excessive body fat represents a serious inefficiency in meat production and may provide health conscious consumers a negative image of the meat industry. Therefore, it is important to develop safe and effective means to facilitate greater production efficiency and improve meat composition in meat animals.

Use of immunological approaches to modulate animal growth and to allow more protein and less fat accretion has attracted recent interest. The use of immunological approaches for modulation of fat deposition in meat animals is still in the early stages of development. However, if proven successful, this approach could provide the meat industry an alternative method for fat reduction. Two possible applications will be discussed in this section. Directly, elimination of fat content has been accomplished by passive immunization with polyclonal antiserum against adipocytes. Indirectly, immunological methods used to manipulate the concentration and activity of growth hormone (GH) has been shown to increase the efficiency of animal production.

Immunological manipulation of growth hormone

When administered to lambs (Wagner and Veenhuizen, 1978), calves (Brumby, 1959), and pigs (Etherton et al., 1987), GH results in a dramatic increase in body protein and a similar decrease in carcass fat,

respectively. However, the use of exogenous hormone treatment has not been accepted by either government regulatory agencies or the public and its practical use may be limited (Muir, 1985). Consequently, the search for alternative methods for improving animal production has continued.

GH release is mainly influenced by two hypothalamic factors: GH-releasing factor, which stimulates GH release, and somatostatin, which inhibits GH release. Antibodies were used to neutralize the functions of somatostatin and consequently to increase the concentration of GH. Varner et al. (1980) have shown that immunization of lambs against somatostatin results in elevation of blood GH, but such an elevation was not accompanied by an improvement in animal growth. However, improvement in animal growth through immunozation against somatostatin has been demonstrated by other researchers (Spencer and Garssen, 1983; Spencer et al., 1983; Laarveld et al., 1986).

A fascinating observation has also been described whereby monoclonal antibodies when complexed to GH, rather than neutralizing its activity, actually dramatically enhanced its potency (Holder et al., 1985; Aston et al., 1986). Human GH/MAb complexes promoted more significant increases in body weight and protein content and a decrease in fat content in treated mice than in equivalent groups treated with human GH alone. In addition, weight gain expressed as g/g food eaten was significantly greater in human GH/MAb animals than in the comparable groups given human GH alone (Holder et al., 1988). Several possibilities were proposed for the mechanism of MAb enhancement of hormone activity, including prolonged association of hormone/receptor complexes or restriction of binding of hormone/MAb complexes to those receptors associated with the enhanced actions (Aston et al., 1986).

Another alternative approach currently under investigation is the use of anti-idiotypic antibodies to GH which can serve as structural and functional mimics of the hormone. Anti-idiotypic antibodies to rat GH were produced and shown to be able to displace specifically ^{125}I-labelled GH from GH receptors in liver and adipose tissue of both rats and sheep. These anti-idiotypic antibodies were also capable of stimulating an increase in body weight gain in hypophysectomized rats (Gardner et al., 1990).

Passive immunization against adipocytes

This approach involves the use of antibodies to adipocytes, which are capable of destroying such cells *in vivo* resulting in considerably reduced body fat deposition. In short-term (Flint et al., 1986) and long-term (Futter and Flint, 1986) trials with rats, injection of antibodies against rat adipocyte plasma membranes reduced body fat deposition. Addition of such antibodies to incubations of isolated adipocytes caused cell lysis. A single administration of antibodies to the rat was effective in reducing carcass fat by 30% without altering feed intake. Carcass protein was also increased significantly (Flint et al., 1986).

Passive immunization against sheep adipocyte plasma membrane reduced perirenal adipose tissue weight and decreased the ether extractable content of both subcutaneous and perirenal fat. Treatment tended to reduce average backfat thickness and estimated kidney pelvic fat (Nassar and Hu, 1991a).

MATERIALS AND METHODS

1. Preparation of plasma membrane fractions

Adipose tissue samples were obtained from inguinal, epididymal, and perirenal fat depots of male Sprague-Dawley rats (160 to 180 g). Adipocytes were isolated by collagenase (Worthington Biochemical Co., Freehold, N.J.) digestion (Rodbell, 1964) at the concentration of 9 mg/g tissue/3 ml Krebs-Ringer bicarbonate buffer (KRB, pH 7.4) containing 4% (w/v) bovine serum albumin and 10 mM glucose. After one hour of incubation at 37°C in a gyratory waterbath, the digested mixture was filtered through chiffon (200 mM diameter) to remove undigested materials, followed by several washes with prewarmed KRB buffer (pH 7.4). Intact mature adipocytes were recovered from the top of the filtrate and resuspended in the prewarmed lysing medium (pH 7.6). The adipocyte suspension was then subjected to vigorous shaking to facilitate cell disruption. Plasma membrane was collected by centrifugation at 30,000 *g* for 30 min. Liver homogenates were prepared according to the methods of Hertzberg (1983). Kidney, spleen, skeletal muscle, and heart homogenates were prepared according to the method of Lo et al. (1976). Plasma membrane enriched fractions were prepared using a self-forming Percoll gradient at 4°C (Belsham et al., 1980). The plasma membrane fractions were washed three times with NaCl-based buffer (pH 7.4) to remove the Percoll. Protein content was measured according to the method of Bradford (1976) using bovine serum albumin as a standard. The isolated plasma membrane fractions were then aliquoted (100 ul/tube) and stored at 4°C. For long term storage the addition of 0.02% sodium azide or freezing was required.

2. Immunizations

Conventional approach. Three female BALB/c mice (6 weeks old) were injected intraperitoneally (i.p.) with 50 mg (200 µl/mouse) of inguinal adipocyte plasma membrane (APM) proteins emulsified in Ribi adjuvant system (RIBI Immunochem Res. Inc., Hamilton, MT). A second 200 µl/mouse i.p. injection (50 mg of inguinal APM protein in Ribi adjuvant) was given on Day 21. On day 26 blood samples were taken from each mouse, serum collected, and anti-APM antibody production was confirmed by enzyme-linked immunosorbent assay (ELISA). A final booster injection (200 µl/mouse) of 50 mg of inguinal APM protein in Ribi adjuvant was administered on day 35 by i.p. injection (Fig. 1).

Cyclophosphamide approach. Female BALB/c mice (6 weeks old) were injected i.p. with a mixture of epididymal and perirenal APM proteins (25 ug each preparation in Ribi adjuvant). Ten minutes later, cyclophosphamide (Sigma Chemical Co.), 100 mg/kg body weight, was given i.p. in saline (2 mg/ml). The same dose of cyclophosphamide was given at 24 h and 48 h following the first injection. This tolerating treatment was repeated every 2 weeks for a total of 4 times. Following a three-week rest, the mice were immunized with the inguinal APM protein (50 ug in 200 µl/mouse). A final booster injection (200 µl/mouse) of 50 ug of inguinal APM protein in Ribi adjuvant was administered two weeks later by i.p. injection (Fig. 2).

3. Fusions

Three days after each final injection, mice were euthanized and spleens removed using sterile techniques. Splenocytes from the hyperimmunized mice were chemically fused with Sp 2/O-AG14 myeloma cells using a polyethylene glycol solution: 40% polyethylene glycol 1450 (Eastman Kodak Co., Rochester, NY), 10% polyethylene glycol 4000 (J. T. Baker Inc., Phillipsburg, NJ), 10% dimethyl sulfoxide (Sigma Chemical Co.), and 40% Dulbecco's modified Eagle's medium (Sigma Chemical Co.), pH 7.2 (Oi and Herzenberg, 1980). After the fusion procedure, cells were resuspended in hypoxanthine-aminopterin-thymidine (HAT) medium with 15% fetal bovine serum (FBS) and plated into 96-well culture plates for the selection of hybridomas. Cells were maintained in HAT medium until macroscopic colonies were observed, and the myeloma controls were dead. HAT medium was then replaced by hypoxanthine-thymidine medium containing 10% FCS, and finally by Dulbecco's modified Eagle's medium-10%FCS (Hyclone Laboratories Inc., Logan, UT). When medium in individual culture wells became acidic (yellow), those wells were tested for anti-APM antibody production by ELISA. Wells containing hybridomas which showed a positive response in ELISA were minicloned and expanded according to the limiting dilution method. Subsequently, wells which contained single hybridoma colonies were subcloned a second time.

4. Enzyme-linked immunosorbent assay (ELISA)

An ELISA method was developed to detect the presence of anti-APM antibodies in culture fluid and to test cross-reactivity of the antibodies

(Nassar and Hu, 1991a). Adipocyte plasma membrane proteins were diluted in PBS to a protein concentration of 3 ug/ml, and 100 µl/well of this preparation was adsorbed onto wells of 96-well microtiter plates (Costar Co., Cambridge, MA) by overnight incubation at 4°C. Plates were then emptied and wells were "blocked" by the addition of 5% nonfat dry milk in PBS (350 µl/well) and incubated at 37°C for 1 hour to prevent nonspecific protein binding. After the blocking step wells were washed once with PBS-Tween (PBS containing 0.05% Tween 20; Sigma Chemical Co.), and 50 µl of hybridoma culture fluid or mouse antiserum was added to appropriate wells. Plates were incubated at 37°C for 1 hour, and the wells were washed four times with PBS-Tween. Each well then received 50 µl of a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule; Sigma Chemical Co.) followed by incubation at 37°C for 1 hour. The plates were washed four times with PBS-Tween and 50 µl/well of substrate solution added. Substrate solution consisting of *P*-nitrophenyl phosphate (Sigma Chemical Co.), prepared at a concentration of 1 mg/ml in glycine buffer (0.1 M glycine, 1.0 mM $ZnCl_2$, 1.0 mM $MgCl_2$, pH 10.4), was added to each well, and the plates were incubated at 37°C for 25 minutes. Absorbance was measured at 405 nm using an ELISA plate reader (Flow Labs, Mclean, VA). For the cross-reactivity studies, the APM coating proteins were replaced by either heart, kidney, liver, skeletal muscle, and spleen plasma membrane proteins (at 0.3 µg/well). For controls, the hybridoma culture fluid was replaced by the fresh culture medium.

5. Immunoblotting

The APM fractions from different adipose tissues were mixed with an equal volume sample buffer which contained 2% SDS, 10% glycerol, 62.5 mM Tris-HCl buffer (pH6.8), 5% 2-β-mercaptoethanol, and 0.002% (w/v) bromophenol blue (Sigma Chemical Co.) and subjected to electrophoresis (1 ug of protein/lane) through a 10% polyacrylamide slab gel according to Laemmli (1970) using a Mini-protean II apparatus (Bio-Rad Laboratories, Richmond, CA). Rainbow protein molecular weight markers (Amersham Co., Arlington Heights, IL) were used to estimate the molecular weight of the separated protein bands. Protein bands were electrophoretically transferred onto a nitrocellulose sheet (0.45mm pore; S&S Inc., Keene, N.H.) using a Genie Blotter (Idea Scientific Inc., Corvallis, OR) according to the method of Towbin et al (1979) as modified by the manufacturer. Blotting was performed for 3 hours at 12V. Detection of the binding activity was accomplished mostly by following the protocol described in VECTASTAIN ABC-AP Kits (Vector Laboratory, INC). Nitrocellulose filters were "blocked" with 0.2% Tween 20 in PBS for 1 hour using continuous shaking (all subsequent incubations were with continuous gentle shaking). Blocked filters were incubated with a 1:200 dilution of hybridoma supernatant in 0.1% Tween20 in PBS for 2 hours and washed three times. Filters were then incubated for 30 minutes with biotinylated secondary antibody solution and washed three time, and this was followed by incubating filters with Vectastain ABC-AP reagent for 30 minutes. Finally, the filters were incubated in the Vectastain ABC alkaline phosphate substrate buffer for approximately 20 min for color formation. The reaction was stopped by rinsing the filters with copious volumes of distilled, deionized water.

6. Immunohistochemistry

Isolated inguinal adipocytes were examined by an indirect immunofluorescence technique (Killefer and Hu, 1990a) with antiadipocyte hybridoma supernatant. Inguinal adipocytes were isolated as described and incubated with a 1:70 dilution of the hybridoma culture fluid in PBS for 30 min at room temperature and washed three times with PBS. The adipocytes were then incubated with a 1:80 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma Chemical Co.) in the dark for 30 min at room temperature. Specimens were observed using a Zeiss epifluorescence microscope fitted with a 35-mm camera. Controls consisted of supernatants from ELISA-negative hybridomas.

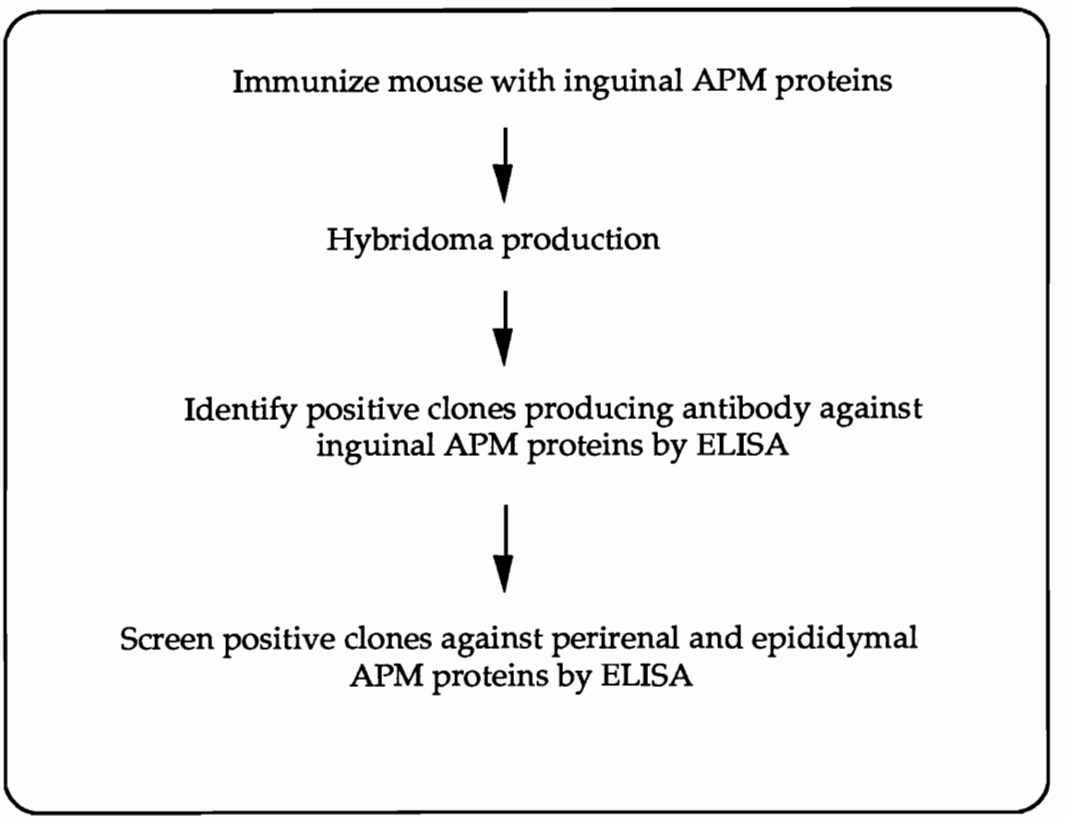

Fig. 1. Conventional approach: Production of a site-specific antibody against inguinal adipocyte membrane proteins

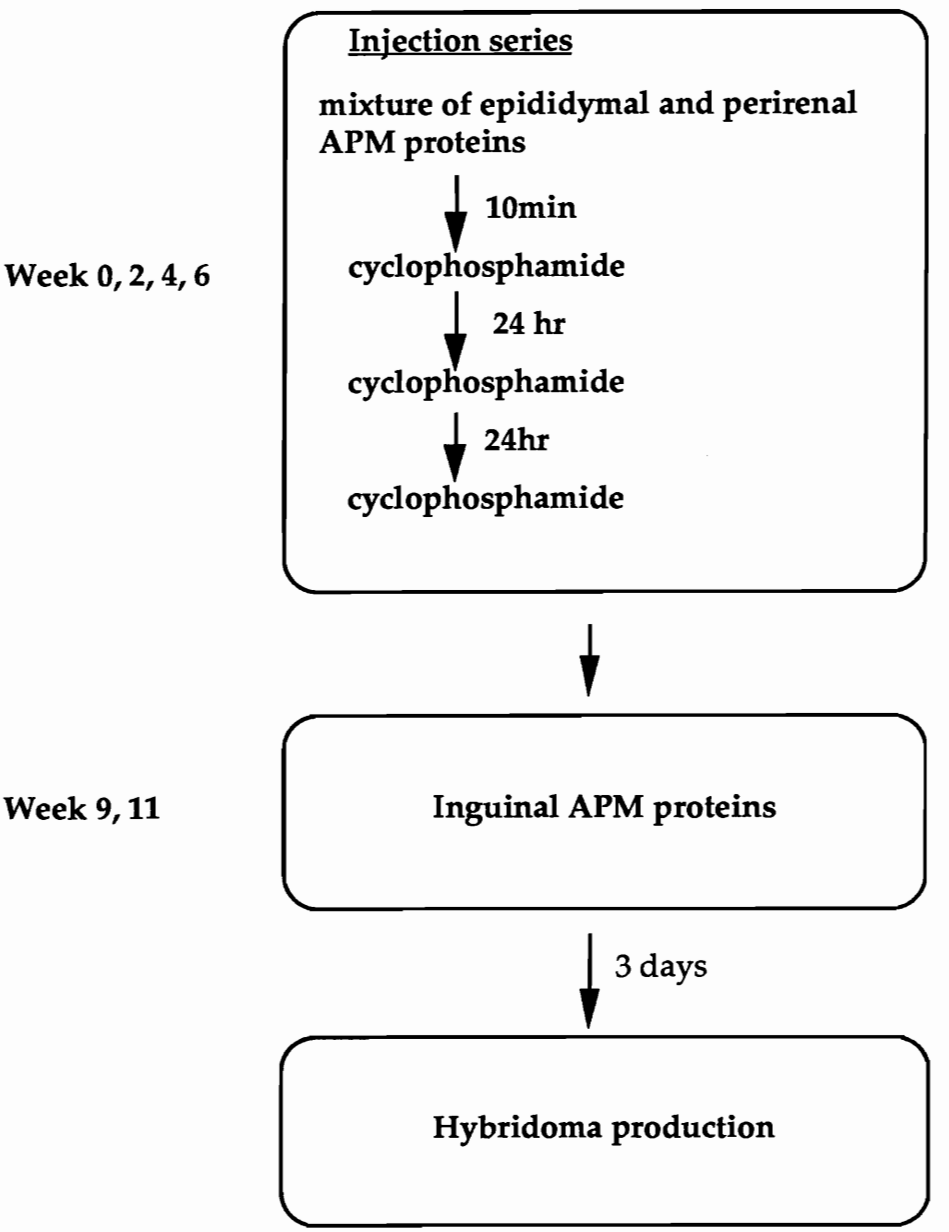

Fig. 2. Selective elimination of clones producing antibodies against immunodominant membrane antigens by cyclophosphamide

RESULTS

Preparation of plasma membrane proteins

A total of 8 mg of inguinal, 5 mg of epididymal, and 2 mg of perirenal plasma membrane proteins was obtained from 112 Sprague-Dawley rats.

MAb production by conventional approach

At the beginning of this study, the conventional protocol (Fig. 1) was used for the purpose of obtaining a MAb specifically against inguinal APM proteins. Three Balb/c mice were used in this study. ELISA was used in the initial screening of the hybridoma supernatant for anti-inguinal APM MAbs. Positive clones were tested for the cross-reactivities to plasma membrane proteins prepared from epididymal and perirenal adipose tissues. Several rounds of ELISA results showed 13 positive clones out of 864 clones screened. After minicloning procedures, 51 positive clones were obtained.

All of the positive clones appeared to react with both epididymal and perirenal APM as strongly as with inguinal APM (Fig. 3). However, ELISA analysis using plasma membrane (PM) proteins prepared from five physiologically distinct tissue types (heart, kidney, liver, muscle, and spleen) demonstrated only low levels of cross-reactivities to some of the positive clones. Figure 3 presents results obtained from one of the clones designated as RA-1. Although RA-1 did not react specifically to inguinal adipocytes, it is still an adipocyte specific MAb.

MAb production by cyclophosphamide approach

Since positive clones produced by the conventional approach failed to recognize any unique proteins in inguinal APM, the cyclophosphamide approach was used to manipulate the direction of the normal immune response and to select for site-specific MAbs (Fig. 2).

All of the six mice used in the first trial of this study died during the immunization procedure. Since Ribi adjuvant was applied for each APM protein injection in this trial, the side effects of adjuvant was considered the most likely cause of death for the mice which became more vulnerable to infection after the injection of cyclophosphamide.

In the second trial adjuvant was not used for the injection. Four of six mice immunized with APM proteins alone died during the immunization procedure. The experiment proceeded with one of the two surviving mice by performing fusion three days after the final injection. Eight positive clones were obtained after minicloning the single positive clone from the primary screening. The other mouse was sacrificed for fusion 6 days after final injection. No positive clone was obtained. Four repeated tolerization steps with cyclophosphamide at 2 week intervals were found to have suppressed antibody production when serum titers to epididymal and perirenal APMs were evaluated. After two subsequent immunizations with inguinal APM, cell fusion was performed on spleen cells from these mice.

ELISA analysis (Fig. 4) indicates that a MAb, RI-1, which has higher binding activity to inguinal APM than to perirenal and epididymal APM, was produced. Significant variations in relative binding activities between APMs of inguinal and the other two sites could be achieved by a serial

dilution from 1:1 to 1:625 of RI-1 MAb culture fluid. Moreover, comparable low levels of cross-reactivities to PM of the same five tissue types used in previous tests were observed (Fig. 4). ELISA demonstrated that significant binding activity between inguinal APM and the RI-1 MAb is still present at the 1:25 dilution of the culture fluid containing MAb, whereas the relative absorbancy of both epididymal and inguinal APM decreased to a level similar to that of heart, kidney, liver, muscle, and spleen PM (Fig. 5). The specificity of RI-1 MAb to inguinal APM improved as the amount of both MAb and coating proteins decreased.

Western blot analysis using RI-1 MAb

For further characterization of RI-1 MAb western blotting technique was applied to detect any possible unique proteins present in the inguinal APM. Inguinal, epididymal and perirenal APM were separated by SDS-PAGE and transfered to nitrocellulose paper. Blots performed with alkaline phosphatase-conjugated goat-anti-mouse IgG antibody revealed a unique protein band with molecular weight at around 25 kDa in the inguinal APM lane but in neither epididymal nor perirenal APM lanes. However, a cross-reactive band of approximately 62 kDa molecular weight exists in all three sites (Fig. 6).

Indirect immunofluorescence using RI-1 MAb

Incubation of RI-1 MAb and fluorescein-conjugated anti-mouse IgG antibody demonstrates that RI-1 MAb appears to bind to intact, isolated inguinal adipocytes. This indicates that the proteins with the specific

antigenic site recognized by RI-1 are actually present on the external surface of the inguinal adipocyte plasma membrane (Fig. 7). No staining was noticed when inguinal adipocytes were incubated with culture fluid of the negative wells from ELISA analysis which served as the negative control (data not shown).

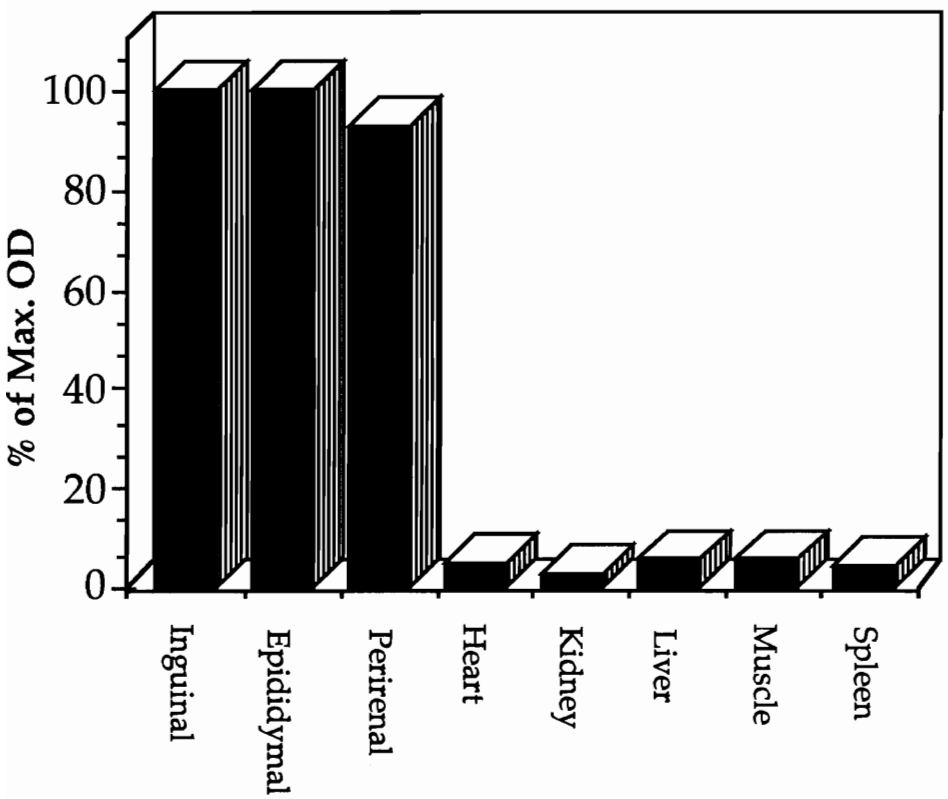

Fig. 3. Relative cross-reactivities of RA-1 MAb to plasma membrane fractions from different tissues assayed by ELISA. Each well in the 96-well microtiter plate was coated with 0.3 μg of plasma membrane fraction from the following tissue types: (1) inguinal, (2) epididymal, and (3) perirenal adipose, (4) heart, (5) kidney, (6) liver, (7) muscle, and (8) spleen. Each was then assayed fo cross-reactivity with RA-1 MAb by ELISA. Fresh cell culture medium was added as the negative control. The relative cross-reactivities are indicated as percentage of the maximal values (100%).

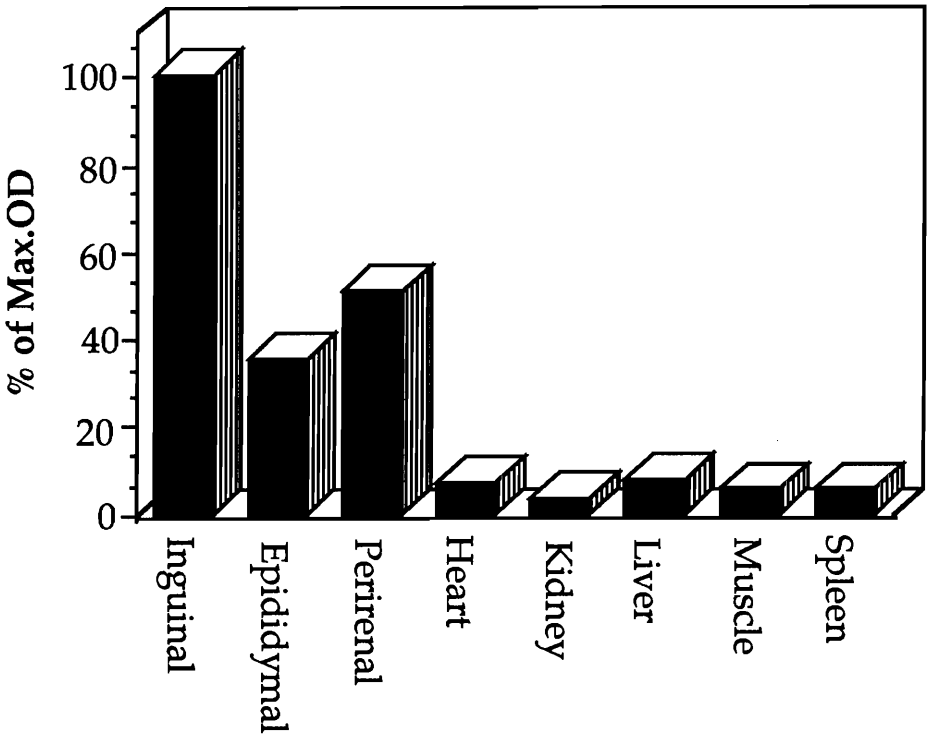

Fig. 4. Relative binding activities of RI-1 MAb to plasma membrane fractions from different tissues assayed by ELISA. Three different adipose tissues: (1) inguinal, (2) epididymal, and (3) perirenal, and five physiologically distinct tissues: (4) heart, (5) kidney, (6) liver, (7) muscle, and (8) spleen were assayed for the binding reactivity with RA-1 MAb. The 96-well microtiter plates were coated with 0.3 μg/well of plasma membrane preparation. Fresh cell culture medium was added as the negative control. The relative cross-reactivities are indicated as a percentage of the maximal values (100%).

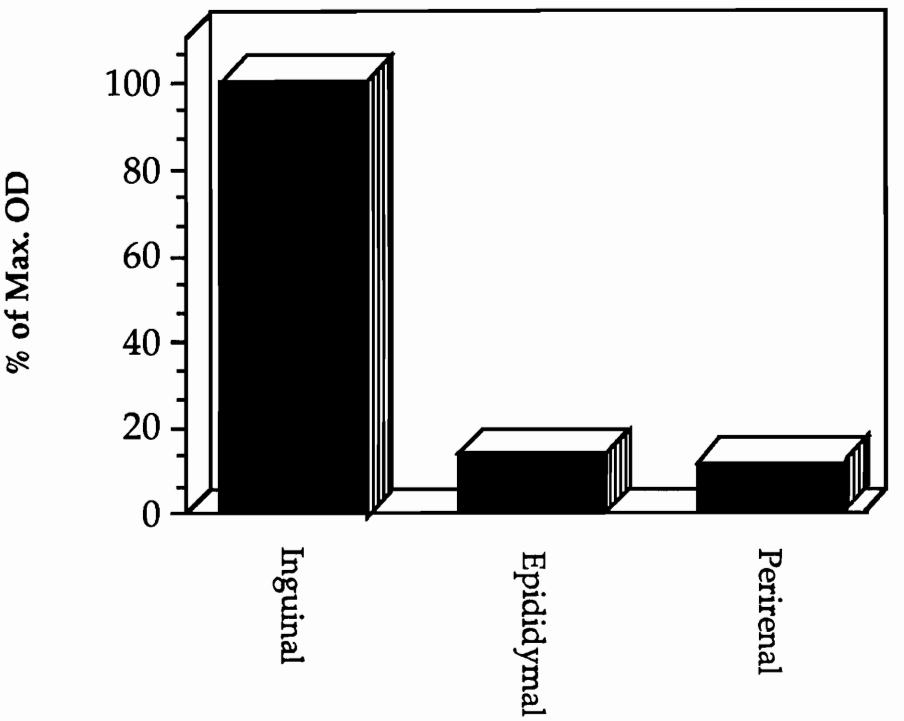

Fig. 5. Relative binding activities of RI-1 MAb to plasma membrane fractions from three different adipose tissues assayed by ELISA. Microtiter plates were coated with 0.1μg of the plasma membrane per well from (1) inguinal, (2) epididymal, or (3) perirenal adipose tissue and assayed for binding activity with culture fluid of RI-1 MAb clone at 1:25 dilution. The relative cross-reactivities are indicated as a percentage of the maximal values (100%).

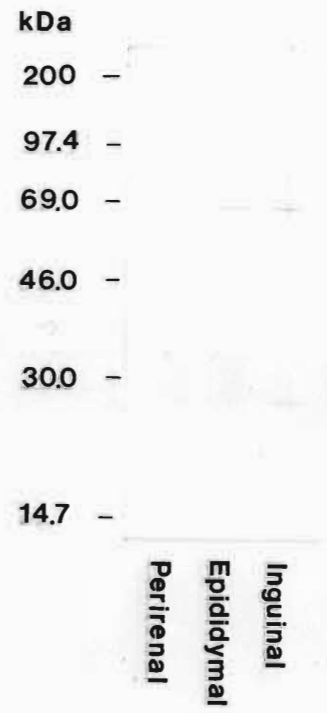

Fig. 6. Western blot analysis of the binding of RI-1 MAb to three different adipose tissues. Plasma membrane from (1) inguinal, (2) epididymal, and (3) perirenal adipose tissues were loaded onto 10% SDS-PAGE gels at a level of 1μg/lane, separated, transfered to nitrocellulose, and immunoblotted with RI-1 MAb.

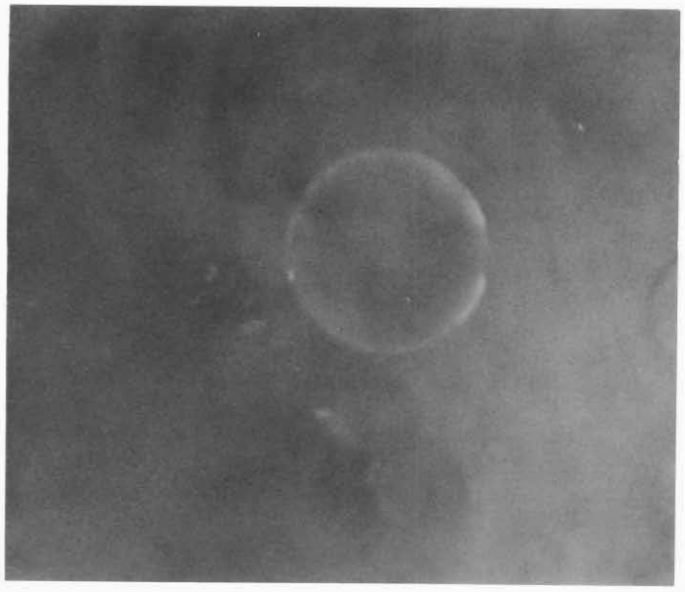

Fig. 7. Indirect immunofluorescence of isolated inguinal adipocytes by RI-1 MAb. Surface antigens were detected by staining with RI-1 MAb culture fluid, followed by FITC-conjugated goat anti-mouse IgG. Proteins reacting with RI-1 MAb were present on the outer surface of the inguinal adipocytes.

DISCUSSION

The specific triacylglycerol-storing function of adipose tissue strongly indicates a unique structure and physiology of adipose tissue. However, adipose tissue per se is not a homogeneously functioning entity. Furthermore, regional differences have been reported (see Literature review). The mechanisms responsible for regional differences in the adipose metabolism have been elucidated only partially. It seems likely that the unique identity and physiological functions of cellular components of adipose tissue are reflected in the enzyme and receptor composition of the cell plasma membranes (Killefer and Hu, 1990a). APM is composed of specific intrinsic, integral, and extrinsic proteins. Study of adipocyte development, morphology, and metabolism has been limited by a lack of characterization of these proteins. Therefore, the identification of site-specific membrane proteins would set the stage for further study of mechanisms of both adipocyte differentiation and development reflecting regional differences between adipose tissues.

Investigation of adipose tissue development has been hindered by the inability to distinguish between morphologically similar cells. Immunological approaches have been successfully used to identify species-specific adipocyte proteins. This approach can distinguish between morphologically similar cells before identification of biological function of antigenic molecules. Furthermore, the use of monoclonal antibodies makes it less necessary to purify the unknown immunogens before immunization. Therefore, monoclonal antibody methodology was adopted in this study to identify any likely site-specific APM proteins. By using cyclophosphamide, a MAb was developed which identified a protein

present specifically in the inguinal APM. This MAb was shown to be of the IgM isotype.

Conditions for western blotting procedures were optimized at the beginning of this study. Electroblotting was performed for 3 hours, based on the outcome of a time-course experiment. In this test, blotting was performed for 2, 3, 4 hours, and overnight respectively. In addition, 10% (v/v) methanol was added to the transfer buffer to increase the transfer efficiency, rather than the 20% (v/v) methanol used by most researchers. In the detection phase to achieve the lowest background, the optimal concentration of each culture fluid was determined. If the culture fluid was too concentrated, high background resulted. This was especially important when using the highly sensitive avidin-biotin antibody detection system. Blocking buffers containing various blocking agents (5% NFDM, 3% BSA, 3% gelatin, and 0.2% Tween20) and two buffer systems (PBS and TBS) were tested. Both 5% NFDM and 3% BSA consistantly gave higher background in both buffer systems than 3% gelatin and 0.2% Tween 20. However, the PBS buffer system produced a lower background than the TBS buffer system. Because 0.2% Tween 20 in PBS buffer had the lowest background and could be prepared more easily than gelatin, it was used in all subsequent experiments.

Immobilon-P transfer membrane (Millipore Co., Bedford, MA), a hydrophobic polyvinylidene difluoride (PVDF) based membrane solid phase for protein blotting was tested as an alternative to nitrocellulose. It offers high mechanical strength and high protein binding capacity but the sensitivity was not different from less expensive nitrocellulose membrane (S&S Inc., Keene, NH), which was used through all subsequent blotting experiments.

Results obtained from ELISA and immunoblotting analysis of 51 positive clones in the first experiment indicated that MAb produced by the conventional approach appeared not to react with any proteins unique to the inguinal APM. The failure of RA-1 MAb to react specifically with the inguinal adipose tissue suggests that proteins shared by inguinal, epididymal and perirenal APMs are likely the immunodominant forms. The difficulty in the conventional approach is that adipose tissues from these three sites are quite similar biochemically and share many immunodominant antigenic determinants. Site-specific plasma membrane proteins may be present, but because they are non-immunodominant, they are unable to induce an antibody response using the conventional approach. A method was needed to improve the probability of detecting subtle antigenic differences.

To overcome this impediment, selective pressure was applied during the immunization stage to preferentially allow the induction of lymphocytes by the presumably non-immunodominant proteins. The immune system responds to any particular antigen with the proliferation of a particular set of lymphocyte clones in a complex manner depending upon the characteristics of each antigen and the immunological history of the animal. Therefore, cyclophosphamide was applied in the next stage of the immunization protocol to selectively kill mitotic cells. Consequently, the immune system is left with a repertoire of lymphocytes capable of response to different antigens in subsequent immunizations. The strategy is based on the assumption that the clonal proliferation of B and T lymphocytes emerges when a particular immune response is elicited; then the dividing cells can be preferentially killed by the alkylating agent, cyclophosphamide.

Because this study was focused on antigens present in the inguinal, but absent in the epididymal and perirenal adipose tissues, homogenates of epididymal and perirenal APMs were applied as the negative immunogens in this case, and inguinal as the positive immunogen. Antibody production was suppressed after four repeated tolerization steps with cyclophosphamide at 2 week intervals.

The production of RI-1 MAb, which later was shown to bind to proteins specifically present in the inguinal APM, was accomplished through the application of cyclophosphamide. The results of the dilution experiments using ELISA (Fig. 5) indicated that 25 fold diluted culture fluid of RI-1 MAb has seven times more binding activity with inguinal than with epididymal and perirenal APMs. Further characterization by western blot illustrated that in addition to the 62 kDa cross-reactive protein band present in all three adipose tissues, a unique 25 kDa protein band exists specifically in the inguinal APM. RI-1 MAb reacts with two proteins, suggesting that these two polypeptides may have a common epitope under the reducing condition employed in the immunoblotting analysis. The alternative hypothesis is that the smaller polypeptide is post-translationally derived from the larger polypeptide. Cleavage of the polypeptide may be due to the presence of inguinal site-specifically expressed proteolytic activity. This result indicates that RI-1 clone is not site specific but rather that it recognizes a unique protein in the inguinal APM. However, the relationship between 62 kDa and 25 kDa polypeptides requires further characterization.

The side effects of adjuvant should be monitored carefully during antibody production in animals. Adjuvant containing killed bacteria can invoke very aggressive and persistent granulomas. All of the six mice

immunized with APM proteins in Ribi adjuvant in the cyclophosphamide approach died during the 6 week immunization procedure. Adjuvant side effects were considered to be a contributing cause for the death of mice.

Four out of six mice immunized with APM proteins alone in the cyclophosphamide approach died during the immunization procedure. Immunosuppressive drugs make animals more vulnerable to infection. It is likely that the death of these mice can be contributed to the strong side effects of cyclophosphamide. Furthermore, the consequence that only one primary positive clone was obtained from the two surviving mice could be due to a generally weakened immune system. In addition, the absence of positive clones in the mice whose spleen cells were fused to myeloma cells 6 days after the final injection with inguinal APM can be explained by the general observation that B lymphphocyte population drops three days after immunization. After minicloning, all the 8 positive clones analyzed have the same characteristics in results from both ELISA and western blotting. This indicates that they are all derived from the same primary clone.

Numerous studies have provided sufficient information to suggest that the growth of adipose tissues varies with the anatomical location under various stimuli. Since the development of adipose tissue involves changes in both the number of mature adipocytes and the capacity of some adipocytes for lipid assimilation (Hirsch et al., 1989), any intra- or extracellular factors involved in these processes can possibly lead to the morphological and biochemical differences present among the adipose tissues at different locations. Identification of a unique protein in inguinal APM may support the possibility that regional distinctions in adipose tissue characteristics imply site-specific expression of genes intrinsic to inguinal adipocytes rather than extracellular influences (Djian et al., 1983). During

embryo development cells are directed by some unknown factors to form different tissues. If adipose tissues at different sites show different expression, this might be the result of tissue development (Lau, 1990). Therefore, the variation observed in the adipose conversion capacity of precursor cells which is strongly dependent on the anatomical site underscores the necessity to carefully interpret the results obtained from only one depot.

The cross-reactivity assessment of RA-1 and RI-1 MAb to other tissue types indicates that both MAbs interact only with proteins whose expression is restricted to adipogenic cell types. Further characterization of the MAbs is necessary. In order to obtain antibodies recognizing specifically the 25 kDa polypeptide, the protein band can be cut off a polyacrylamide gel and subsequently used as an immunogen. If cross-reactivity still occurs, cyclophosphamide-accompanying immunization with the 62 kDa protein band may be used preceding the 25 kDa protein immunization to enhance the desired immune response. These monoclonal antibodies will provide defined and reproducible reagents for study of the development of adipose tissue at the different anatomical locations using the unique 25 kDa protein as a specific marker.

Indirect immunofluorescent studies indicate that RI-1 MAb recognizes a protein located on the surface of the cytoplasmic membrane (Fig. 7). However, whether 62 kDa , 25 kDa or both polypeptides are responsible for the fluorescence observed remains to be investigated. Additional characterization of the proteins may be pursued initially by a similar study using intact epididymal and perirenal adipocytes. Also, it remains to be determined whether this protein is also present intracellularly. Since adipocytes are prone to be broken at lower

temperature, this experiment was not performed at 4°C, which is generally used to decrease the metabolic activity of the intact cells. The identification of the site-specific protein and its physiological function, which are most intriguing, will be the focus of future studies.

CONCLUSION

The studies presented in this thesis demonstrate the production of a adipocyte-specific monoclonal antibody using a traditional approach without cyclophosphamide selection during immunization. Furthermore, a monoclonal antibody recognizing a 25 kDa unique polypeptide in the inguinal APM was selectively raised through the application of cyclophosphamide, which preferentially eliminated dividing lymphocytes during immunization with both epididymal and perirenal APM proteins. Further characterization of the unique protein will be required. Production of an antibody reacting specifically to the 25 kDa inguinal adipocyte-specific protein first needs to be accomplished. The availability of 25 kDa-protein-specific-antibody will allow us to study the biochemical function and structure of the protein, and to identify the specific biological role of the protein in inguinal adipose tissue development

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APPENDICES

A. Isolation of plasma membrane proteins from rat adipocytes

I. Adipose Cell Isolation
II. Adipose Cell Ghost Preparation
III. Plasma Membrane Isolation

Protocol:

I. Adipose Cell Isolation:

1. Slice tissue 0.025" (0.65mm) thick or mince finely.
2. Add 15 ml KRB-Glu-BSA (37°C) to a 25 ml polypropylene flask or siliconized glass flask. (3 ml buffer/gram tissue)
3. Add 6,000 units (approx. 45mg) Type I collagenase (Worthington, Freehold, NJ) to the flask. (3 mg/ml buffer, 2,000 units/ gram tissue), (Weighing paper works better than weighing boats to get all off)
4. Swirl flask to dissolve collagenase.
5. Gas medium (gas phase only!) with O_2:CO_2 (95:5) for 10 min., stopper.
6. Add 5 g tissue.
7. Incubate 1 hour at 37°C in a gyratory water bath, setting 2 (New Brunswick scientific, Edison, NJ) "60 min. for Perirenal, 70 min. for Epididymal, 90 min. for Inguinal"
8. Filter through chiffon into a polypropylene tube with cap. Carefully squeeze cells through chiffon with a polypropylene rod and add 2 ml of KRB-HEPES.
9. Place centrifuge tube in 37°C water bath and let cells rise to top.
10. Remove infranatant with siliconized needle and syringe. Discard infranatant.
11. Slowly add about 10 ml KRB-HEPES (2-3 ml/g tissue) and cap tube, gently invert to wash cells.
12. Discard infranatant and wash two more times.
13. Remove infranatant and resuspend in sucrose extraction medium. (10 ml)
14. Now have isolated adipocytes.

(Note: Step 13 may be omitted if proceeding directly with lysis of the adipose cells. Proceed with step 1 of Fat Cell Ghost Preparation).

II. Fat Cell Ghost Preparation

1. Remove the infranatant from the cell suspension and add 10 ml prewarmed (37°C) lysing medium (2 ml/g tissue).
2. Adipose cells are disrupted by shaking the capped tube vigorously by hand, agitating using a vortex-mixer, or repipetting through a #23 gauge needle.

3. Place centrifuge tube in 37°C water bath and let intact cells rise to top.
4. Aspirate the infranatant beneath the fat cells into a cold polypropylene test tube (on ice).
5. Repeat lysing procedure 3 times more adding 10 ml, 5 ml, and 5 ml prewarmed lysing medium respectively.
6. Centrifuge at 4°C, 30,000 X g for 30 minutes (16 K rpm in Beckman JA-17).
7. Decant and discard supernatant and wipe the test tube wall free of lipid, using a finger covered with a Kimwipe.
8. Now have the pellet.

III. Plasma Membrane Protein Isolation

1. To the pellet add 1-2 ml cold sucrose extraction medium (4°C) and vortex to thoroughly resuspend.
2. Combine all tubes.
3. Gently apply 800 ul of the pellet suspension to the top of an 8 ml Percoll gradient in a clear polypropylene tube that fits the Beckman JA-20.1 rotor.
4. Centrifuge at 10,000 X g for 15 minutes at 4°C to stratify (11K rpm in JA-20.1 outer ring).
5. Remove infranatant leaving approximately 2 ml below the top band and discard.
6. Combine the contents of the two tubes into a 50 ml polypropylene tube.
7. Wash with 3X volumes of cold NaCl-based medium (4°C).
8. Centrifuge at 30,000 X g for 10 min. at 4°C (16K rpm, JA-17).
9. Discard supernatants and resuspend the plasma membrane pellets in NaCl-based medium.
10. Mix all together in one tube and wash one more time.
11. Resuspend the pellet in the minimum of NaCl-based medium.
12. Run protein analysis using dye binding assay.
13. Can be stored at 4°C, long term storage may require addition of 0.02% sodium azide, freezing may be possible.

Media:

1. Preparation of KRB (Krebs-Ringer-Bicarbonate) - 20 mM HEPES and KRB-Glu-BSA buffer (0.03M Glucose, 4% BSA in KRB-HEPES buffer), containing 100 µg/ml streptomycin-penicillin.

KRB-HEPES		KRB-Glu-BSA	
130.0 ml	1000 ml	130.0 ml	1000 ml

0.770M NaCl	20.0 ml	7.110 g	20.0 ml	7.110 g
0.770M KCl	0.8 ml	0.362 g	0.8 ml	0.362 g
0.770M KH_2PO_4	0.2 ml	0.163 g	0.2 ml	0.163 g
0.770M $MgSO_4.7H_2O$	0.2 ml	0.295 g	0.2 ml	0.295 g
0.770M $NaHCO_3$	4.2 ml	2.148 g	4.2 ml	2.148 g
0.300M Glucose	13.0 ml	5.400 g	13.0 ml	5.400 g
H_2O	87.1 ml	990 ml	61.1 ml	800 ml
0.275M $CaCl_2.2H_2O$	0.6 ml	0.194 g	0.6 ml	0.194 g
1.000M HEPES	2.6 ml	4.770 g	2.6 ml	4.770 g
Strep-Pen solution	1.3 ml	10.0 ml	1.3 ml	10.0 ml

Adjust pH to 7.4
Gas 10 min. with O_2:CO_2 (95:5) before adding BSA

20% solution BSA Fraction V	26.0 ml	200 ml

2. Lysing Medium (1000 ml)

		weight component
2.5 mM	ATP (2Na +$2H_2O$)	1.4680 g
2.5 mM	$MgCl_2.6H_2O$	0.5080 g
0.1 mM	$CaCl_2.2H_2O$	0.0147 g
1.0 mM	$KHCO_3$	0.1000 g
2.0 mM	Tris base	0.2420 g

Bring to volume with d.d. H_2O
Adjust pH to 7.6 with HCl
* ATP is not required if preparation are not used for enzyme activity studies.

3. Sucrose Extraction Medium (500 ml)

		weight component
0.25 M	Sucrose	42.7880 g
10.00 mM	Tris base	0.6060 g
2.00 mM	EGTA	0.3810 g

Bring to volume with d.d. H_2O
Adjust pH to 7.4 with HCl

4. Sucrose-Tris-EGTA (20 ml)

		weight component
2.0 M	Sucrose	13.692 g
80.0 mM	Tris/HCl	0.252 g
8.0 mM	EGTA	0.600 g

Bring to volume, no pH adjust

5. Iso-Osmotic Percoll Solution

7	parts of Percoll
1	part of Sucrose-Tris-EGTA
32	parts of Sucrose Extraction Medium

* Keep Percoll stock solution sterile.

6. NaCl-based Medium (500 ml)			weight	component
150	mM	NaCl	4.384	g
10	mM	Tris base	0.606	g
1	mM	EGTA	0.191	g
20	mM	HEPES	2.383	g
	Strep-Penn		5.000	ml

Bring to volume
Adjust pH to 7.4 with HCl

B. Isolation of plasma membranes from rat heart, kidney, liver, muscle, and spleen.

Protocol:

All steps are at 4°C

1. Tissue is cut into small pieces with scissors.
2. 2.0 g tissue in 30 ml ST buffer.
3. Homogenize 25 sec at speed 7 using polytron (Brinkmann, Westbury, NY).
4. Centrifuge at 1,500 g for 15 min. (4000rpm, JA-17, Beckman Model J2-21)
5. Decant off supernatant.
6. Resuspend the pellet well in 3 ml ST buffer.
7. Add 30 ml Iso-Osmotic Percoll Solution - Mix well.
8. Centrifuge at 10,000 g for 20 min. (11K rpm, JA-17)
9. Using syringe and needle remove infranatant leaving about 5 ml below top band (PM band).
10. Transfer PM band to a new tube and add 30 ml ST buffer.
11. Centrifuge at 1,500 g for 10 min. (4,000 rpm, JA-17)
12. Discard supernatant and resuspend pellet in 30 ml ST buffer.
13. Centrifuge at 1,500 g for 10 min. (4,000 rpm, JA-17)
14. Discard supernatant.
15. Resuspend the pellet in minimum of NaCl-based medium.

Reagents:

1. ST buffer (pH 7.4): 0.25 M Sucrose, 10 mM Tris/HCl, 2 mM EGTA

2. Sucrose-Tris-EGTA (no pH adjust): 2 M Sucrose, 80 mM Tris/HCl, 8 mM EGTA

3. Iso-Osmotic Percoll Solution:
   - 7 parts of Percoll
   - 1 part of Sucrose-Tris-EGTA
   - 32 parts of ST buffer

4. NaCl-based medium (pH 7.4):
   - 150 mM NaCl
   - 10 mM Tris/HCl
   - 1 mM EGTA
   - 20 mM Hepes
   - 1 ml/100ml medium Strep-Pen (10 mg/ml)
   - or 1 ml/1 L medium Gentamycin (50 mg/ml)

C. Production of monoclonal antibody

I. Immunization of mice
II. Hybridoma formation
III. ELISA screening

I. Immunization of mice

1. 50 ug PM/mouse/injection (Ribi adjuvant system).
2. Inject day 0, boost day 21, trial bleed day 26; if titers O.K., boost day 35 and take spleen for fusion 3 days after boost.

II. Hybridoma formation

1. Preparation of spleen cells for fusion
2. Preparation of mouse myeloma cells for fusion
3. Hybridization
4. Minicloning by limiting dilution

1. Preparation of spleen cells for fusion

1. Sacrifice an immune Balb/c mouse and put into beaker of 95% ethanol for 5-10 seconds.
2. Position mouse with its right side down on alcohol soaked paper towels.
3. Using 2 rat toothed forceps, carefully tear the skin layer open.
4. Dip the tips of forceps in ethanol , carefully open the muscle layer in the same manner and remove the spleen.
5. Put the spleen in a 60 mm petri dish containing 5 ml filter sterilezed Dulbeco's MEM-0 (DME-0).
6. Using two #23 gauge needles, gently tease the spleen apart into very fine pieces.
7. Transfer the spleen material into a sterile 15 ml conical tube and let set to allow large pieces to settle.
8. Pipette the cell suspension into a sterile 15 ml tube and bring the volume up to 10 ml with DME-0.
9. Centrifuge 8 minutes at 400 X g at room temperature and remove the supernatant.
10. Resuspend the cell pellet in 10 ml DME-0.
11. Centrifuge 8 minutes at 400 X g at RT (repeat for a total of 3 times)
12. Remove the supernatant from final wash.

2. Preparation of mouse myeloma cells for fusion

Myeloma cells are grown in DME-10 (10% Fetal Bovine Serum).

1. Dump out old culture fluid from the flask.

2. Add 10 ml DME-0 to the flask and "RAP" cells loose.
3. Transfer the cell suspension to a 15 ml sterile centrifuge tube (1 flask/tube).
4. Centrifuge 6 minutes at 400 X g at room tempetature.
5. Decant off supernatant.
6. Add 10 ml DME-0, repipette carefully to suspend cells.
7. Centrifuge and wash two more times.
8. Remove the supernatant from the final wash.

3. Hybridization

1. Mix spleen and myeloma cells in a 50 ml conical centrifuge tube in 20 ml DME-0. but before doing this:
   - Compare the packed cell volumes of spleen cells and myeloma cells
   - Want equal amounts (or more spleen cells if necessary)
   - Can adjust volumes for resuspending suspension, so this is accomplished in 20 ml total DME-0, setting aside excess suspension.
2. Centrifuge 8 minutes at 400 X g at room temperature to pellet cells together.
3. Remove the supernatant , being certain to remove all fluid.
4. Add 1 ml PEG over 1 minute, using an Eppendorf repeatly pipette with a 2.5 ml tip extended with a yellow pipet tip.
5. Countinously stir 1 minute longer using same tip.
6. Let sit 1 minute.
7. Stir in 1 ml DME-0 over 1 minute.
8. Stir 1 minute more.
9. Let sit 1 minute.
10. Stir in 1 ml DME-0 over 1 minute.
11. Let sit 1 minute.
12. Stir in 7 ml DME-0 over 3 minutes.
13. Let sit 1 minute.
14. Centrifuge 8 minutes at 400 X g at room temperature.
15. Discard the supernatant and resuspend fused cells in 30 ml HAT.
16. Plate out into 3 sets of 96-well culture plates (100 ul/well).
17. As a control, the parent (unfused) myeloma cells are plated in 3 wells (100 ul/well).
18. Add 100 ul HAT to each well.
19. Incubate the cultures at 37°C, 5% CO_2.

4. Minicloning by limiting dilution

1. Prepare the following 4 tubes with the appropriate volumes of DME-0 or DME-10.

Tube 1	2.5 ml	DME-0
Tube 2	4.5 ml	DME-0
Tube 3	5.0 ml	DME-10

Tube 4 5.0 ml DME-10

2. Suspend the cells by using a 100 ul pipette and transfer approximately 1-2 drops of the suspension to Tube 1. Examine a drop of the diluted suspension microscopically to confirm the presence of cells. Need 6-10 cells in the field of entire depth (100X).
3. Mix the cells in Tube 1 by agitation and transfer 1 ml of the suspension to Tube 2. Agitate Tube 2 and transfer 1 ml of this suspension to Tube 3. Agitate Tube 3 and transfer 1 ml to Tube 4.
4. Transfer the appropriate number of drops of cell suspensions from Tube 3 and 4 into the 96-well microplate as indicated below:

Tube		1	2	3	4	5	6	7	8	9	10	11	12
3	A	2	2	2	2	2	2	2	2	2	2	2	2
3	B	2	2	2	2	2	2	1	1	1	1	1	1
3	C	1	1	1	1	1	1	1	1	1	1	1	1
3	D	1	1	1	1	1	1	1	1	1	1	1	1
4	E	2	2	2	2	2	2	2	2	2	2	2	2
4	F	1	1	1	1	1	1	1	1	1	1	1	1
4	G	1	1	1	1	1	1	1	1	1	1	1	1
4	H	1	1	1	1	1	1	1	1	1	1	1	1

5. Incubate the plates in the CO_2(5%) incubator at 37°C until clonal growth is visible. Many of the wells should contain only one colony, suitable for expansion to larger culture volumes.

III. Screening (Enzyme Linked Immunosorbent Assay, ELISA)

1. Coat the 96-well microplate (Costar No. 3590) with 100 ul/well of the coating agent (eg 2.5 ug/ml APM) and incubate at 37°C for 2 hours or overnight at 4°C.
2. Empty the plate and add 350 ul/well of blocking solution (5% Non-Fat Dry Milk in PBS), incubate at 37°C for 1 hour.
3. After incubation period empty the plate and wash once with PBS-Tween 20 (0.05%). Add 50 ul of culture fluid/well, incubate at 37°C for 1 hour.
4. Empty the plate and wash 4 times with PBS-Tween 20. Pound dry on a paper towel and pop all bubbles.
5. Add 50 ul/well of goat anti-mouse-alkaline phosphatase conjugate diluted 1:1000 in PBS, incubate at 37°C for 1 hour.
6. Empty the plate and wash 4 times with PBS-Tween 20. Pound dry on a paper towel and pop all bubbles.
7. Add 100 ul/well of enzyme substrate solution (1 mg *P*- Nitrophenyl Phosphate/ml) to each well.
8. Incubate at 37°C for 20-30 min. in the dark.
9. Read the optical density at 405 nm in an ELISA plate reader (Flow Labs, Mclean, VA).

Media:

1. Phosphate Buffered Saline (PBS):

	Conc. (mM)	Per L (g)
KH_2PO_4	2.85	0.39
K_2HPO_4-$3H_2O$	7.15	1.64
NaCl	150.00	8.50

pH to 7.4

2. Substrate Solution:
   a. Stock solution:

1.00 M Glycine (10X)	7.507 g/100 ml
0.05 M $ZnCl_2$ (50X)	0.682 g/100 ml
0.05 M $MgCl_2$ (50X)	1.017 g/100 ml

Aliquot and freeze

   b. working solution: (10 ml)

		Conc.
1.00 M Glycine	1.0 ml	100 mM
0.05 M $ZnCl_2$	0.2 ml	1 mM
0.05M $MgCl_2$	0.2 ml	1 mM
d.d. H_2O	8.6 ml	

Adjust pH to 10.4 using 1 N NaOH
Add 1 mg/ml *P*- Nitrophenyl Phosphate (Sigma No. 104-0)

D. Western analysis of monoclonal antibodies

I. Separation of proteins
II. Electroblotting of proteins to nitrocellulose
III. Antibody reaction

Protocol:

I. Separation of proteins:

1. Set up the SDS-PAGE mini-gel according to the manual provided by manufacture (Bio-Rad).
2. Boil samples to be loaded for 4 min. Briefly spin the samples to bring liquid to the bottom.
3. Load samples into wells using a Hamilton syringe or pipettor.
4. Run the gel at 200V for about 45 min.

II. Electroblotting of proteins to nitrocellulose

1. Assemble the apparatus as follows from botton to top: Make sure the components are tightly assembled.
2. a. Two bubble screens
   b. Cathode (banana connector in upper left)
   c. One bubble screen (ribbed side down)
   d. A Scotchbrite pad
   e. Add transfer buffer
   f. Two sheets filter paper cut to size (Whatman #1)
   g. Electrophoresis gel
   h. Pre-wetted nitrocellulose
   i. One sheet filter paper
   j. Two Scotchbrite pads
   k. One bubble screen (ribbed side up)
   l. Anode and plastic anode cover
3. Transfer at 12V for 3 hours at 4°C.

III. Antibody Reaction (all steps done at room temperature, shaking)

1. Wash the membranes for 1 min. in PBS.
2. Incubate in blocking solution (0.2 % Tween 20 in PBS) for 1 hour.
3. Wash for 5 min. in TPBS.
4. Primary Ab incubation: Incubate with dilution of antibody in TPBS for 2 hours.
5. Wash 3X for 5 min. in TPBS.
6. Secondary Ab incubation: Incubate with biotinylated secondary Ab in TPBS for 30 minutes.

7. Wash 3X for 5 min. in TPBS.
8. Incubate with Vectastain ABC-AP reagent (Vector, Cat. No. AK-5002) in TPBS for 30 minutes.
9. Wash 3X for 5 min. in TPBS.
10. Color reaction: Use Alkaline Phosphatase Substrate Kit II. (Vector, Cat. No. SK-5200). Immediately before use, add two drops of reagent 1 to 10 ml of the buffer (100 mM Tris-HCl, pH 9.5) and mix well. Add two drops of reagent 2, mix well and finally add two drops of reagent 3 and mix well.
11. Stop with d.d. H_2O (4 X 1 min.) when color is at desired intensity.
12. After washing, photograph for a permanent record. Colors may fade on the original blots.

Reagents:

1. Transfer buffer (no pH adjustment necessary)

	Conc.	Per liter
Tris/base	25 mM	3.03 g
Glycine	192 mM	14.40 g
Methanol	10%(v/v)	100 ml

2. Phosphate Buffered Saline (PBS)

	Per L (20X)	Per L (1X)
KH_2PO_4	7.8 g	0.39 g
K_2HPO_4-$3H_2O$	32.8 g	1.64 g
NaCl	170.0 g	8.50 g
pH = 7.5		

3. TPBS: 0.1 % (v/v) Tween 20 in PBS