Vascularisation in adipose depots surrounding immune-stimulated lymph nodes

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ABSTRACT

We report a change in the vascularisation of the adipose depots surrounding the popliteal lymph node that has, and the contralateral node that has not, been exposed to a simulated immune challenge. The percentage of the depot that consists of vessels, as measured by image analysis, decreases over a period of 2 d after immune stimulus, then increases in a biphasic manner over the next 2–3 wk. By 1 mo after the stimulus, the vascularisation has returned to baseline values. The adipose tissue surrounding both the stimulated and the unstimulated lymph nodes shows a similar pattern, but the unstimulated depot lags by 3–6 d in reaching its maximum vascularisation. These data support the hypothesis that perinodal adipose tissue is involved in peripheral immune responses.

Key words: Lymphatic tissue; immune activation; perinodal adipose tissue.

INTRODUCTION

We have used in vivo and in vitro methods to demonstrate interactions between lymph node lymphoid cells and the adipose tissue surrounding lymph nodes (Pond & Mattacks, 1995; Mattacks & Pond, 1997). Most recently we have studied the popliteal adipose tissue and lymph node of the rat (MacQueen & Pond, 1998). The popliteal lymph node has been used for many years as a marker of lymph node activation in graft-versus-host and host-versus-graft reactions (Twist & Barnes, 1973; Sanderson et al. 1995). In this essay, the lymph node itself becomes redder and increases in mass as it is activated. While examining the appearance of receptors to tumour necrosis factor-α (TNFα) after a simulated immune challenge to the rat popliteal lymph node (MacQueen & Pond, 1998), we observed that the adipose tissue around the activated lymph node appeared pinker than in an unstimulated depot, suggesting that increased blood perfusion involved the adipose tissue as well as the node within it. This is a novel observation, since in spite of the long standing and widespread use of the popliteal lymph node for studying peripheral immune responses, it is always excised from surrounding tissues before examination. Studies on the blood perfusion of adipose tissue have recently been reviewed (Crandall et al. 1997). In general, the flow rate through adipose tissue, as in lymph nodes, is low. Adipose tissue itself produces angiogenic factors in a depot-specific pattern. Furthermore, adipocytes can produce adenosine and prostaglandins, which can result in vasodilation, and angiotensinogen, which, via angiotensin II, can result in vasoconstriction.

Bacterial lipopolysaccharide (LPS) is known to induce the synthesis of TNFα (Carswell et al. 1975), which has been reported to be angiogenic (Frater-Schroeder et al. 1987; Leibovich et al. 1987). Popliteal adipocytes have some receptors for TNFα, whether or not the lymph node has been stimulated, but more appear within a few hours of activation (MacQueen & Pond, 1998): it seems likely, then, that our experimental protocol for eliciting receptors to cytokines may also increase the number of vessels in the adipose depot surrounding a stimulated lymph node. In this study we have used image analysis to measure directly the amount of vascular tissue in the popliteal adipose depot surrounding stimulated and unstimulated lymph nodes over time.
MATERIALS AND METHODS

Rats were CFHB (Wistar-derived) males, aged between 8 and 9 wk, and of body mass 350–450 g, at the time of injection. They were bred at the Open University, kept on a 14 h d–10 h night cycle and fed RM3 diet. The rats appeared to be in good health, but were not raised in specific pathogen-free conditions. A total of 35 rats were used in this study.

The left popliteal lymph node was activated by injecting subcutaneously lipopolysaccharide (Sigma UK, Poole) in phosphate-buffered saline (PBS), pH 7.4, at approximately 1 µg per 100 g body mass into the lower left hind limb, i.e. distal to the node. The injected volume was 0.08 ml. This treatment produced no apparent discomfort to the animal, and no subsequent discolouration or swelling in the injected limb could be discerned.

At various times after the simulated immune challenge rats were killed by cardiac injection of 1.0–1.5 ml of 60 mg/ml sodium pentobarbitone (Sagatal, Rhône-Mérieux, Ireland) and the entire popliteal adipose depots, each containing its single lymph node, were dissected immediately from each hind limb, weighed, and fixed in formol calcium fixative (2 mM calcium chloride in 10% formalin, pH 7.2). They were kept at +4°C until sectioned.

Sectioning was done using a Vibratome Series 1000 (General Scientific, Redhill, UK), with a nominal section thickness of 120 µm. This thickness, being 20% more than the diameter of the largest adipocytes (Bjorntorp et al. 1982), permitted good visualisation of the material. Sectioning was carried out on a bed of dry ice to solidify the tissue. Slides were precoated with poly-L-lysine, and sections were mounted in PBS, pH 7.2.

Whole adipose depots, containing the popliteal lymph node, were sectioned and every section of every depot was then analysed using a Magiscan (Applied Imaging, Sunderland, UK). Using a ×4 objective, images were projected onto a screen and all the visible vessels outlined using a light pen. The area bounded by the light pen, corresponding to the area of vessel in that field, was then measured by the processor (Fig. 1). The areas of the sections themselves were measured in a similar way. The total areas of vessels and of the whole sections were noted. Since the whole depot was examined, the percentage that was occupied by vessels—the vascularised area—could then be calculated.

Confocal microscopy was carried out using a Leica TCS-NT confocal microscope fitted with a Krypton-Argon laser (Leica, Milton Keynes, UK). The fluorescent sections were viewed under ×10 magnification to locate the vessels. After locating a vessel, the magnification was increased to ×40 and the
section was viewed confocally using the standard FITC filter system. Optical sections were obtained at 0.5 µm intervals and stacked using the confocal software to give a maximum projection image. The images were annotated, sized and printed using Adobe Photoshop 4.0. To improve visualisation for confocal microscopy only, tissue slices were stained with FITC-labelled *Ulex europaeus* agglutinin I (Vector Laboratories, Peterborough, UK, catalogue number FL-1061).

**RESULTS**

Figure 2 shows the change in percentage of the popliteal adipose depot occupied by vessels in both the stimulated leg and the unstimulated leg at various times after a local immune challenge. Each point represents the mean of data from between 2 and 5 animals, each depot of which provided more than 100 measured fields. The depots from both legs showed an initial decrease in the vascularised area which was most marked on the first day following the injection, but decreased still further on the second day. However, by the third day the vascularised area had returned to slightly above the baseline value, and this increased until d 5. A fall on d 6 was followed by an increase to d 14 in the stimulated leg. In the unstimulated depot, the fall on d 6 was maintained until d 7, and the subsequent rise lagged behind that seen in the stimulated leg, although a similar maximum was reached at d 20. The difference between the stimulated and the unstimulated legs is statistically significant (*t* test for matched samples). By d 29, the vascularised area in both depots had returned to baseline values.

In each section there were, in addition to larger blood vessels visibly filled with erythrocytes, smaller thin-walled structures which appeared largely empty. Before including the latter in the measurements, attempts were made to establish whether they were blood capillaries, lymph vessels, or artefacts. Both capillaries and lymph vessels are lined with endothelium, which can be identified by various immunological markers. However, in our system the reagents also reacted positively with adipocytes, so were not deemed suitable for unequivocal identification of endothelium (data not shown). Cross-reactivity between endothelium and adipose tissue has been reported previously, and is thought to be due to the common developmental lineage of these 2 tissues (Wright & Hausman, 1990).

Confocal microscopy allowed us to follow the paths of these small structures with more accuracy. Under higher magnification it was clear that they were indeed vessels. A few appeared to be lymphatic vessels arising de novo within the adipose tissue, as they were very...
narrow and had ‘blind’ ends as shown in Figure 3a; they all showed a tubular structure (Fig. 3b). The majority were clearly blood capillaries, joining more major blood vessels, and sometimes containing erythrocytes, as shown in Figure 4. Measurement of the vessels supported our identification of them as lymph vessels or capillaries. The blind-ended tube in Figure 3a has an outer diameter of 4–5 μm, and the inner diameter of the tubular structure shown in Figure 3b is approximately 8 μm. These values are consistent with previously reported measurements (Crandall et al. 1997). Furthermore, the erythrocyte shown in Figure 4 has a diameter of 6–7 μm, which is generally accepted as a typical value (Vander et al. 1994). There was no evidence that anything we might tentatively identify as a vessel would turn out not to be one, and we were thus confident in including these small tubular structures in our measurements.
DISCUSSION

Angiogenesis is believed to be an important part of the physiological response to a bacterial infection. In a rabbit corneal implant system, vascular sprouts were first seen 2 d after TNFα implantation. Inflammatory angiogenesis proceeded rapidly, but the blood vessels started to regress about 14 d after implantation (Frater-Schroeder et al. 1987). The time course that we observed in the adipose tissue surrounding the stimulated lymph node is consistent with this report, although the initial reduction in vessel area, and the biphasic increase are differences that may derive from the different experimental systems used. Interestingly, Hay & Hobbs (1977) reported a transient drop in both mass and blood flow in the rabbit popliteal lymph node over the first 2 d following immune challenge, an observation consistent with that which we have made here. The regression of vessels that we observed after 14 d in the stimulated depot, and after 20 d in the unstimulated one, is not surprising in view of earlier findings, but we cannot say with certainty whether we are seeing blocking of perfusion of vessels which remain in place, or a net reduction in vessel number. Adipose tissue has the known capacity to generate all these effects (Crandall et al. 1997).

In contrast to the changes in receptors reported previously, which are most extensive in the adipose tissue surrounding the stimulated lymph node and most marked over the first 24 h after immune challenge, the vascular response occurs also in the homologous tissues of the unstimulated leg, and in broadly the same time frame in both legs. In vitro experiments have shown that adipose tissue from all the major depots that enclose lymph nodes, though not that from the perirenal depot, which lacks nodes, has the capacity to respond to stimuli from activated lymphoid cells (Pond & Mattacks, 1995). This response is enhanced by noradrenaline (Pond & Mattacks, 1998). If the vascular responses that we see in the depots surrounding both the stimulated and the unstimulated lymph nodes have a systemic trigger, it might be mediated by the sympathetic nervous system. The sympathetic nervous system is known to mediate the vascular effects of insulin (Scherrr & Thornbury, 1990). We do not know whether other blood and lymph vessels elsewhere in the body also constrict and then dilate during this period, although this remains a strong possibility. Given our injection protocol, which elicited molecular events that occurred rapidly around the stimulated node only (MacQueen & Pond, 1998), we feel that stimulation of systemic responses due to leakage of LPS from the site of injection is unlikely; however, we cannot completely exclude this possibility.

We have suggested that perinodal adipose tissue is specialised to respond to secretions from lymph node lymphoid cells, and stored materials then released supply and regulate the local immune response so that it is prompt and adequate, but avoids becoming systemic if a peripheral reaction is sufficient to deal with the immune challenge (Pond, 1996a, b; MacQueen & Pond, 1998). It is conceivable that the initial vasoconstriction that we see relates to this process: because cytokines such as TNFα can have severe effects systemically, there may be good reason for retaining them locally as far as possible. We have shown that receptors for TNFα in the adipose tissue around the popliteal lymph node appear within 30 min of a simulated immune challenge (MacQueen & Pond, 1998). They could mediate local interactions between lymph node lymphoid cells and the perinodal adipose tissue, as postulated by Pond (1996a, b). Adipose tissue itself can produce TNFα, which could amplify the response and enable it to spread rapidly throughout the popliteal depot. However, the changes in these receptors have largely disappeared before the increase in vascularisation reported here begins. Nevertheless, the initial decrease in vascularisation occurs in both legs within 24 h, so some early signal of immune challenge, whether hormonal or nervous, has been communicated within this time scale. It is possible that short term, local effects may include the generation of a longer term ‘amber alert’ that is conveyed throughout the body. The subsequent vasodilation and/or angiogenesis may be associated with a β-adrenergic trigger, and may facilitate the supply of materials to lymphoid cells throughout the body, thus enabling the response to subsequent immune challenges to be faster and/or more extensive. The biphasic pattern that we observed may represent a response to different requirements at different stages.

In conclusion, we have directly measured the vascularisation in rat popliteal adipose depots surrounding lymph nodes which have, or have not, been exposed to a simulated immune challenge. Changes in vascularisation seen over the month after stimulation can be related to the physiological interactions
between lymph node lymphoid cells and the perinodal adipose tissue. These observations support the hypothesis that perinodal adipose tissue is specialised for local interactions with lymph node lymphoid tissue.

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