Interferon Gamma Regulation of *De Novo* Protein Synthesis in Human Dermal Fibroblasts in Culture Is Anatomic Site Dependent

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The propensity of the skin of the lower anterior leg to be involved in Graves' dermopathy prompted an examination of the specific protein synthesis and response to interferon gamma in cultured fibroblasts from this area. Confluent cultures from normal skin of the lower leg and from the abdomen of the same three donors were pulse labeled with [^{35}S]methionine for 3 h and subjected to two-dimensional protein gel electrophoresis and fluorography. Protein spots were mapped using a computer-driven program and the relative densities of the resolvable spots analyzed. Fibroblasts from the two anatomic sites display distinct patterns of *de novo* protein synthesis. Of the 157 abundant spots arbitrarily chosen for analysis, 31% varied substantially in levels of expression between the sites. A number of proteins appear to be expressed only in cultures derived from one of the two anatomic sites. Interferon gamma (100 U/ml) present in the culture medium for 48 h influenced the abundance of a number of proteins in a site-specific manner. Among them, plasminogen activator inhibitor type-1 was induced three to five times in the leg cultures, whereas this same polypeptide was down-regulated in abdominal fibroblasts. A 54-kD protein was induced in interferon-treated cultures from both sites at least 50 times. It appears that fibroblasts from different regions of the integument are intrinsically distinct in terms of both their protein synthetic programs and their responses to cytokines. *J Invest Dermatol 100:288–292, 1993*

Considerable evidence suggests that cultured fibroblasts eminating from different human organs behave in specialized and perhaps unique ways. For instance, gingival fibroblasts respond to the addition of diphenylhydantoin into culture medium with an increased collagen synthetic rate, whereas dermal fibroblasts are unaffected [1]. The accumulation of glycosaminoglycan in lung and orbital fibroblast but not in dermal fibroblast cultures is stimulated by interferon gamma [2,3]. Dermal fibroblasts [4–6], but not those derived from the human orbit [7], respond to 3,5,3′-L-triiodothyronine and glucocorticoids in regard to hyaluronate synthesis. Even among dermal fibroblasts, anatomic site-specific differences in cellular metabolism have been reported. For instance, genital fibroblasts metabolize androgens differently than do those obtained from deltoid or abdominal skin [8].

A satisfactory explanation has yet to be offered for the involvement of discrete dermal regions in systemic diseases such as Graves' disease, in which an autoimmune hyperplastic goiter is in some cases associated with ophthalmopathy and dermopathy [9]. The dermopathic process is usually confined to the skin of the anterior shin [9]. Pretibial fibroblasts, but not those from the prepuce or the skin of the back, respond to sera of patients with dermopathy associated with Graves' disease [10]. Those sera may contain a protease-sensitive, heat-stable, dialyzable factor distinct from 7 S gamma globulin that could stimulate hyaluronate accumulation selectively in the pretibial fibroblasts. Thus, there is reason to believe that pretibial fibroblasts may differ from those eminating from other regions of the integument.

In this paper, we describe studies involving the two-dimensional gel electrophoretic analysis of cell-layer–associated fibroblast proteins from dermal cultures of pretibial and abdominal origin. The results suggest that the protein synthetic programs in fibroblasts from the two distant anatomic regions are distinct. Moreover, interferon gamma influences the synthesis of specific proteins in these fibroblast strains in a site-dependent manner.

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Abbreviations:

FBS: fetal bovine serum
HLA-DR: human leukocyte antigen-DR
IEP: isoelectric focusing
PAI-1: plasminogen activator inhibitor type-1
SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis

MATERIALS AND METHODS

Cell Culture Primary fibroblast cultures derived from normal tissue of individuals with Graves' disease or those without thyroid dysfunction were initiated from dermal biopsies as described previously [11]. The Albany Medical College Institutional Review Board approval has been obtained for these activities. Monolayers were allowed to proliferate to a state of confluence in 35-mm diameter plastic culture plates covered with Eagle's medium containing 10% fetal bovine serum (FBS), glutamine, and antibiotics. Cultures were

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Table I. Protein Synthesis in Cultured Human Dermal Fibroblasts Derived from the Leg and Abdomen

Culture Conditions[a]	Acid-Insoluble cpm ($\times 10^{-5}$)[b] (calf)	(abdomen)
Control	74.6 ± 9.7[c]	73.9 ± 12.1
INF-gamma	67.0 ± 11.5	66.0 ± 11.5

[a] Cells were grown to confluence then exposed to nothing (control) or interferon-gamma (INF-gamma; 100 U/ml) for an additional 2 d.
[b] Cultures were labeled for 3 h in FBS-/methionine-free medium containing [^{35}S]-methionine as described under *Materials and Methods*. Cells were lysed in first-dimension buffer and TCA-insoluble radioactivity determined by filtration.
[c] Mean ± standard deviation.

maintained in a humidifed, 5% CO_2 incubator at a constant temperature of 37°C. The experiments used fibroblasts of a similar passage number, which was always less than 15. Some cultures were treated with interferon gamma for 48 h. The growth media were removed, and labeling medium containing FBS, methionine-free RPMI 1640 medium, and [^{35}S]methionine, specific activity >1100 Ci/mmol (50 μCi/ml) (New England Nuclear, Boston, MA) without (control) or with the cytokine was added. Metabolic labeling proceeded for 3 h.

Two-dimensional Protein Gel Electrophoresis Labeling media were collected and clarified by centrifugation at 13,000 × *g*. Cell lysates were prepared in first-dimensional buffer (9.8 M urea, 2% Nonidet P-40, 2% pH 7.9 ampholytes, 100 mM dithiothreitol). [^{35}S]methionine-labeled cellular proteins (5×10^5 to 2×10^6 cpm) were separated by isoelectric focusing (IEF) on pre-run 1.5 mm diameter tube gels (pH 5-7/3-10 ampholytes, 5 : 1, v/v) before molecular mass separation by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Labeled proteins were detected by fluorography and quantitated densitometrically with a Bioimage gel scanner (Milligen Corp) interfaced with a SUN SPARC work station or with a Zeiss MOPS III image analyzer [12]. Proteins were identified according to their pI/M_r coordinates using the REF52 data base [13]. Each datum point included in Tables II, III, and IV represents the mean ± SD of density analysis in triplicate of protein spots of three gels, each derived from a different donor's culture strain.

RESULTS

Comparison of Specific Protein Synthesis in Cultured Leg and Abdominal Dermal Fibroblasts Cultured human dermal fibroblasts actively incorporate [^{35}S]methionine into many discrete proteins, several hundred of which are sufficiently abundant to be resolved on two-dimensional protein gel electrophoretic fluorograms. Although the various strains did not differ in regard to the uptake of radiolabeled amino acid precursor or its conversion to acid-insoluble material (Table I), the pattern of *de novo* protein synthesis in pretibial skin fibroblasts varied substantially from cultures of abdominal skin (Fig 1). The data contained in Table II are from an analysis of the protein gels depicted in Fig 1 and strains from two additional donors. When 157 arbitrarily chosen spots were quantitated densitometrically and compared, a number were more or less abundant, depending on the anatomic source. Of these spots, 106 were expressed equally in leg and abdominal fibroblasts. The data from 35 representative spots are presented in Table II. Proteins expressed at invariant levels in leg and abdominal cultures include fibronectin *(spot 1)*, collagen *(spot 2)*, vimentin *(spot 91)*, and actin *(spot 114)*. Of the remaining spots, 24 proteins expressed in the leg varied from −93% to +sixfold with respect to their

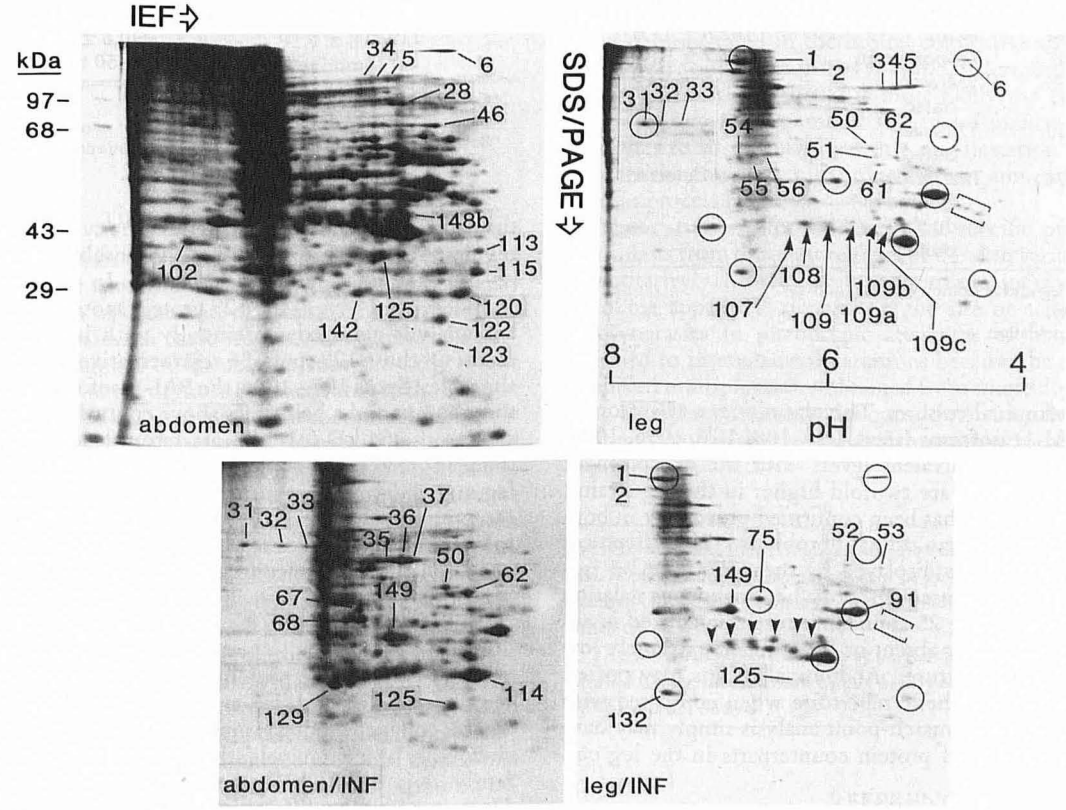

Figure 1. Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled proteins from human fibroblasts in culture. Confluent cultures from abdominal and pretibial skin were treated for 2 d without (control) or with human recombinant interferon gamma (IFN; 100 U/ml) and then labeled for the final 3 h. Samples were prepared as described in *Materials and Methods*. Gels (10% polyacrylamide) were then dried and subjected to autofluorography for 1 week at −70°C followed by scanning densitometry. The *large circles* surround protein spots used to standardize the scanning procedure. *Brackets*, vimentin degradation products. Spot numbering was arbitrary.

Table II. Quantitation of the Relative Abundance of 35 Major Proteins Expressed by Human Dermal Fibroblasts[a]

Spot Number	pI/M_r Coordinates	Tentative Identification	Relative Abundance ×100[b] (OD leg/OD abdomen, in percent)
1	6.7/220	Fibronectin	110.9 ± 12.6[c]
2	6.8/190	Collagen	90.3 ± 13.1
6	4.9/230	Unidentified	364.7 ± 42.4
10	5.6/130	Unidentified	104.0 ± 8.0
11	5.4/130	Unidentified	146.3 ± 31.2
16	6.8/107	Unidentified	355.0 ± 66.6
18	6.3/100	Unidentified	≤5
19	6.2/100	Unidentified	≤5
20	5.6/100	Unidentified	88.3 ± 9.7
27	5.4/94	Alpha-actin	91.4 ± 7.6
34	7.0/93	Unidentified	19.2 ± 10.2
37	6.2/92	Unidentified	75.9 ± 11.8
39	6.5/91	Unidentified	117.4 ± 22.3
42	5.6/87	Unidentified	18.1 ± 6.5
43	5.5/86	Unidentified	≤5
47	8.0/90	Unidentified	77.7 ± 9.2
51	5.6/78	Unidentified	≤5
59	6.2/70	Unidentified	≤5
91	5.1/57	Vimentin	94.4 ± 18.3
97	7.0/57	Unidentified	198.3 ± 72.1
98	7.3/56	Unidentified	576.5 ± 109.6
99	7.5/55	Unidentified	420.7 ± 99.9
107	6.2/46	PAI-1	212.0 ± 51.0
108	6.1/46	PAI-1	144.3 ± 32.5
109	5.9/46	PAI-1	91.1 ± 21.7
109a	5.8/46	PAI-1	230.4 ± 19.6
109b	5.7/46	PAI-1	100.1 ± 11.5
109c	5.6/46	PAI-1	80.7 ± 10.3
110	4.9/55	Vimentin degradation	111.8 ± 16.4
113	4.9/41	Tropomyosin	30.3 ± 15.6
114	5.2/43	Actin	102.8 ± 11.0
115	4.8/37	Tropomyosin	11.7 ± 5.3
116	4.9/35	TM-associated	109.8 ± 17.2
119	5.0/30	TM-associated	157.4 ± 38.1
149	5.9/54	Interferon-gamma inducible protein	Not detected

[a] Derived from the leg and abdomen of three donors.
[b] OD, optical density.
[c] Mean ± standard deviation.

Table III. Effects of Interferon Gamma on Specific Protein Synthesis in Dermal Fibroblasts from the Leg of Three Donors

Spot Number	Relative Abundance ×100 (OD Interferon/OD control, in percent)[a]
1	67.8 ± 13.4[b]
2	11.8 ± 4.6[c]
6	426.0 ± 96.0
10	27.0 ± 13.1
11	10.2 ± 3.2
16	99.3 ± 13.9
18	Not detected
19	Not detected
20	21.3 ± 16.5
27	30.4 ± 14.7
34	266.3 ± 63.1
37	339.9 ± 63.7[c]
39	102.5 ± 18.3
42	220.1 ± 23.2
43	Not detected
47	187.2 ± 45.5
51	Not detected
59	Not detected
91	113.7 ± 24.2
97	434.6 ± 107.1
98	47.9 ± 10.3
99	33.5 ± 10.9
107	344.2 ± 93.7
108	506.2 ± 197.3
109	499.1 ± 158.2
109a	386.0 ± 149.9
109b	390.7 ± 63.3
109c	305.5 ± 97.8
110	42.8 ± 16.7
113	206.7 ± 25.4
114	102.6 ± 7.6
115	36.3 ± 8.3[c]
116	79.3 ± 10.1
119	90.6 ± 10.8
149	>50 times

[a] OD, optical density.
[b] Mean ± standard deviation.
[c] Based on data from two strains.

counterparts in abdominal cultures. The plasminogen activator inhibitor type-I (PAI-1) isoforms (spots 107, 108, 109, 109a, 109b, 109c) are expressed at equivalent levels with the exception of spots 107 and 109a, which are twofold higher in the leg strains. The identity of these spots has been confirmed previously in both rodent kidney [14] and human orbital fibroblasts [12]. Differences in the charge characteristics displayed by the various PAI-1 isoforms are thought to be a consequence of a heterogeneous sialation pattern [12]. The remaining 25 abundant proteins analyzed in abdominal cultures were either absent or expressed at extremely low levels (≤5%) in the leg cultures. Abdominal strains may possess a more diverse protein synthetic repertoire when compared with the leg cells. Alternatively, match-point analysis simply may have failed to detect a number of protein counterparts in the leg cultures.

Anatomic Site-Dependent Differences in Interferon Gamma Induction of Protein Synthesis in Dermal Fibroblasts Interferon gamma treatment alters the synthetic rates of several abundant proteins expressed in dermal fibroblast cultures and resolvable on two-dimensional protein gel electrophoresis. As Fig 1 and the data in Tables III and IV suggest, the cytokine can up- or down-regulate specific protein synthesis in fibroblast cultures, depending on the anatomic region of derivation. In dermal cultures derived from leg, only 14% of the 129 protein spots suitable for densitometric analysis changed substantially with interferon gamma treatment. Of those 129 spots, 35 representative proteins are included in the tables. Spots 107–109c, the PAI-1 isoforms, were induced from three to five and a half times above control levels. Spot 149, representing the 54 kD (pI-5.9) interferon-dependent protein [15] was induced greater than 50 times (relative to background levels) in both leg and abdominal cultures but was undetectable in cultures not receiving the cytokine. Spot 6 (M_r 230, pI 4.9) was induced (fourfold) in the leg cultures as were spots 37 (3.4 times), 97 (4.3 times), and 47 (1.9 times). In contrast, spot 2 (M_r 190, pI 6.8), a collagen, was down-regulated dramatically to 12% of the level seen in controls. Synthetic rates of spots 10, 11, and 20 were also inhibited 70–90%.

Many of the same proteins appeared to be influenced by interferon gamma in both leg and abdominal skin fibroblasts. For instance, collagen (spot 2) is inhibited in strains from both anatomic sites, more or less equivalently. Spots 37 and 47 are induced equivalently as is the 54-kD interferon-dependent protein (spot 149). However, striking differences in the response to interferon gamma were also noted. The PAI-1 isoforms in the abdominal strains were down-regulated 45% to 94% below control levels, compared with the marked up-regulation in the leg cultures. Likewise, spot 97 was down-regulated twofold in the abdomen, in contrast to the substantial induction in leg fibroblasts.

Table IV. Effects of Interferon Gamma on Specific Protein Synthesis in Dermal Fibroblasts from the Abdomen of 3 Donors

Spot Number	Relative Abundance ×100 (OD Interferon/OD control, in percent)[a]
1	41.7 ± 12.9[b]
2	≤5
6	97.6 ± 8.3
10	98.4 ± 19.2
11	60.5 ± 20.1[c]
16	367.6 ± 51.5
18	363.8 ± 99.6
19	24.3 ± 15.9
20	239.0 ± 38.5
27	104.4 ± 20.3[c]
34	48.8 ± 18.6
37	387.2 ± 91.5
39	>6 times
42	108.3 ± 19.7
43	105.1 ± 10.4
47	383.7 ± 62.8
51	296.2 ± 90.5
59	90.2 ± 15.6
91	107.5 ± 16.1
97	33.4 ± 11.7
98	29.0 ± 8.8
99	43.7 ± 20.1
107	66.1 ± 19.9
108	39.5 ± 18.7
109	Not detected
109a	25.1 ± 3.3[c]
109b	50.5 ± 10.6
109c	≤5
110	56.3 ± 31.8
113	507.5 ± 153.6
114	102.3 ± 14.1
115	59.5 ± 20.2
116	91.7 ± 10.3
119	92.4 ± 8.9[c]
149	>50 times

[a] OD, optical density.
[b] Mean ± standard deviation.
[c] Based on data from two strains.

DISCUSSION

Differences in the pattern of specific protein synthesis found in fibroblasts from distant anatomic regions of the integument may explain the distribution of cutaneous manifestations characteristic of certain systemic diseases. In these studies, we examined the response to interferon gamma of fibroblasts from the skin of the anterior shin as representative of those cells activated in the context of Graves' dermopathy. When compared with abdominal skin fibroblasts, those from the leg demonstrated a substantial induction of PAI-1, whereas abdominal PAI-1 expression was down-regulated to levels below detectability. The 54-kD protein (pI 5.9) known to be inducible in most interferon gamma responsive cell types [15] was induced in cells from both sites equally.

The differential response of PAI-1 to interferon gamma may have implications in the context of inflammatory processes involving the skin. Because that polypeptide is an important determinant of the pericellular proteolytic environment [16], the up-regulation in the leg-derived fibroblast strains may increase matrix stability and thus favor accumulation of extracellular proteins. Those proteins and hyaluronate appear to be important components of the poorly characterized amorphous material accumulating in pre-tibial myxedema lesions [17, 18]. We have already proposed that the profound induction by interferon gamma of PAI-1 in orbital fibroblast cultures [12] coupled with the apparently site-specific stimulation of glycosaminoglycan accumulation in those cultures [3] may be relevant to the pathogenesis of Graves' ophthalmopathy.

The studies reported here involved fibroblasts derived from two sites in each of three donors. In all instances, interferon gamma induced PAI-1 synthesis substantially in the leg fibroblasts, whereas expression of the polypeptide in abdominal cultures was decreased by the cytokine. It is as yet unclear whether the up-regulation of PAI-1 in leg cultures seen here will be universal for all strains obtained from the shin. Moreover, it is uncertain whether the down-regulation observed in the abdominal fibroblast strains is characteristic of all cultures obtained from the torso. Many more strains from these and other anatomic sites will need to be examined before any such conclusions can be made. What does emerge from these results is that, within an individual donor, PAI-1 expression and regulation by cytokines in dermal fibroblasts varies from one anatomic site to another.

Interferon gamma can alter the transcriptional rates of specific genes in a variety of cell types [19]. Many of the nuclear actions of this cytokine are thought to be mediated through its binding to a membrane-bound receptor [20] and the activation of the protein kinase C cascade [21]. Proteins whose synthetic rates are altered in response to interferon gamma include that encoded by the class II major histocompatibility gene human leukocyte antigen DR (HLA-DR) [21,22]. The expression of HLA-DR is ordinarily limited to lymphoid tissues; however, many cell types synthesize this antigen-presenting protein when exposed to interferon gamma. We have reported that fibroblasts of dermal and orbital connective tissue origin respond to the cytokine in this manner and that the induction can be modulated by other cytokines [23] and glucocorticoids [24]. In the current studies, we do not demonstrate a prominent interferon-inducible protein that would correspond to HLA-DR at 63 kD. This is likely the consequence of the relative short treatment periods used in this study (48 h) as compared to those necessary to detect immunoquantifiable HLA-DR antigen in studies using these fibroblasts (96–120 h) [23].

There is reason to suspect that interferon gamma may be a pathogenic factor in Graves' disease. HLA-DR is aberrantly expressed in thyrocytes of involved glands [25,26]. Moreover, the antigen has been demonstrated in the orbital connective tissue from a patient with severe ophthalmopathy [27] and in dermopathic skin [23]. The expression in those tissues is most likely the consequence of the action of interferon gamma. The recruitment of activated T lymphocytes to tissues involved in a manifestation of Graves' disease implies the potential role for interferon gamma in the pathogenesis of that process [17].

These studies offer evidence that specific protein synthesis in fibroblasts from different regions of the skin varies qualitatively and quantitatively. Moreover, response to a major class of inflammatory cytokine appears to depend on the site of cellular origin. These idiosyncrasies in phenotypic attributes of fibroblasts cannot be ascribed to interpersonal variations because the comparisons made involved multiple strains obtained from single donors. Our findings may be of importance to the question of why the skin of the leg is uniquely vulnerable to the inflammatory cascade associated with Graves' dermopathy.

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