Clonal Characteristics of Cutaneous Scars and Implications for Atherogenesis

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Atherosclerotic plaques in man have monoclonal characteristics. One possible explanation for this observation is that clonal selection occurs as cells proliferate at sites of intimal injury. To test whether such clonal selection could occur during the formation of an intimal "scar", the clonal characteristics of human cutaneous scars were studied with the use of glucose-6-phosphate dehydrogenase (G-6-PD) as a cellular marker. Scars from 7 black women heterozygous for the A and B isoenzymes of G-6-PD were divided into 870 portions consisting of contiguous normal skin, scar margin, outer scar, and inner scar. Portions were further divided into surface and deeper portions of scar. In 4 of the 7 cases, significant differences were observed between the mean percentage of total activity in the B isoenzyme band in portions of scar and portions of skin. Significant differences between surface and deeper portions of both skin and scar were also observed in 5 cases. The variance of isoenzyme values from portions of scars was not significantly greater than that within portions of skin. It is concluded that the results are consistent with clonal selection occurring during the healing of skin wounds. However, the magnitude of the difference (no greater than a 5.7% B isoenzyme difference) is far too small to even partially explain the marked differences in isoenzyme patterns observed between atherosclerotic plaques and uninvolved aortic wall. The results do not support the idea that the monoclonality of human atherosclerotic plaques is due to clonal selection following the healing of an intimal injury. (Am J Pathol 1981, 102:49–54)

ATHEROSCLEROTIC PLAQUES have been shown to consist of cell populations derived from a single clone of cells.[1,2,3] The mechanism by which these monoclonal cell populations arise is unknown. The two most likely possibilities include 1) mutation in smooth muscle cells and 2) clonal selection following intimal injury and repair. The latter proposed mechanism has been supported by evidence from *in vitro* studies, which have shown that skin fibroblast cultures contain cells of varying growth potential and that serial cultures select clones with the greatest ability to grow.[4] The authors of these studies predict that healing wounds might be composed of a monoclonal or oligoclonal cell population. However, *in vivo* studies of clonal selection within healing wounds have not been performed. In the present investigation we have determined the clonal characteristics of healed cutaneous scars from women heterozygous for the A and B isoenzymes of G-6-PD, an X-linked enzyme used as a cellular marker. Using methods similar to those employed to determine the clonal characteristics of atherosclerotic plaques, we set out to see whether this process of clonal selection occurs during a simple healing process *in vivo*, thus indicating that a similar process may account for the monoclonality of atherosclerotic plaques.

Materials and Methods

Cutaneous scars and surrounding skin from black females were collected from the autopsy service of The Johns Hopkins Hospital and stored at −70 C for later assay. The age of the patient and age of the scar were noted when possible. Tissue samples from patients found to be heterozygous for the A and B isoenzymes of G-6-PD were then thawed for further dissection. Prior to dissection, a map of the scar and skin was made by tracing the outline of the scar onto

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61.4	59.8	63.4	62.8	57.8	57.4	58.8	66.0	64.2	63.5	SKIN
62.5	60.0	64.3	63.6	63.1	59.7	60.4	55.5	64.9	64.5	MARGIN
63.4	65.1	68.4	59.5	58.7	60.2	57.9	64.6	64.1	64.2	OUTER SCAR
60.8	65.6	65.5	61.8	58.3	62.3	61.8	62.0	65.0	63.0	INNER SCAR
62.1	63.1	67.0	61.1	59.5	59.1	63.6	62.6	65.8	66.9	INNER SCAR
62.5	67.7	69.1	61.8	55.8	60.7	61.8	65.1	65.3	68.4	OUTER SCAR
60.3	64.0	64.8	60.9	65.3	62.0	57.3	61.5	66.7	68.3	MARGIN
65.0	62.1	62.8	60.0	56.0	59.4	56.6	62.9	63.1	62.0	SKIN

Figure 1—The scar from Case 7 is shown, illustrating the manner in which it has been divided into portions. The various types are labeled on the right side; the areas of normal skin are at the top and bottom. The portions designated margin contained both normal skin and scar. The numbers within each portion are the % B isoenzyme value for the upper portions (lower portions' values not shown).

a transparent overlay in order to locate the position of each portion of skin and scar assayed. All adipose and loose connective tissue was then dissected away, leaving the full thickness of skin and scar. Skin more than .5 cm away from the scar was removed. The skin and scar were then sectioned in "bread loaf" fashion at 2-mm intervals perpendicular to the long axis of the scar. These transverse sections were then further divided perpendicularly from both sides of the scar, adjacent skin, scar margin (including both skin and scar), outer scar (next to scar margin), and inner scar (if the size of the scar permitted) (Figure 1). These pieces were then subdivided parallel to the skin surface into upper half (surface half) and lower half (deeper half). Each portion was a cube measuring approximately 2 mm on each side. In some cases the scar was so large that it was impractical to assay the entire scar.

The methods of cellulose acetate electrophoresis and specific enzyme staining for the assay of G-6-PD isoenzymes have been previously described.[2,3] The results were expressed as the percentage of total isoenzyme activity in the B isoenzyme band.

Results were analyzed by calculation of means and standard deviations of the percentage of B isoenzyme for portions grouped according to type and location of portions. The Student *t* test was used to test for significance of differences between means.[5] Analysis of variance, using one-way classification of samples of unequal size, was performed to determine the sources of variation within portions of each type of scar.[5] The *F* test was used to test for significance of differences between variances from two types of portions; Bartlett's test was used to test for significance of differences between variances from several types of portions.[5]

Results

Scars from 7 black women heterozygous for G-6-PD isoenzymes were studied (Table 1). All scars were well healed and 5 or more years old. All but one were obtained from the skin of the abdomen.

Values obtained from G-6-PD assay for each portion could be assigned to the portion's position on the map of the scar; an example is given from Case 7 (Figure 1). Portion-to-portion variation was observed within and between types of portions, but no definite trends could be identified (Figure 2). Very minor differences were observed in the means of portions from various parts of the specimen (eg, inner scar, outer scar, margin, skin, etc), and the variance (as reflected by the standard deviation) was similar for portions from the various parts. There is little evidence of a trend toward a higher or lower value of G-6-PD, and none of the values of portions of scar meets the rigorous criteria used to define monoclonality in atherosclerotic plaques.[3] Portions of the underside of the scar had consistently lower values than portions of the scar surface.

Table 1—Characteristics of Scars Studied

	Patient age (yrs)	Scar location and cause	Scar age (yrs)	Maximum width (mm)
Case 1	52	Abdomen—cholecystectomy	5	16
Case 2	25	Abdomen—splenectomy	10	14
Case 3	67	Abdomen—hemicolectomy	14	7
Case 4	51	Abdomen—hysterectomy	19	9
Case 5	59	Knee—trauma	Unknown	8
Case 6	54	Abdomen—hysterectomy	11	6
Case 7	50	Abdomen—jejunectomy	5	14

Results from all 7 cases are summarized in Table 2. A total of 870 portions were assayed. Not a single portion of scar met the criteria for monoclonality, defined as being *outside* ± 3 SD (99.7%) confidence limits around the mean of the reference tissue (skin) *and* being *within* previously defined 95% confidence limits (less than 25.3% B and greater than 89.2% B) for samples of leiomyomas, known monoclonal lesions.[3] The differences between means of various types of portions within each case were small, the maximum difference being 5.7% B isoenzyme. Nevertheless, due to the relatively small variance of samples and large number of samples, significant differences ($P < .05$) could be demonstrated between means of skin and/or margin and scar in 4 of the 7 cases (2,4,6,7). In three of these cases (2,4, and 6) there was one portion of outer scar (top in Cases 2 and 4, bottom in Case 6) that had an isoenzyme value that fell outside the 99.7% confidence limits for normal skin but did not fall within the 95% confidence limits for leiomyomas listed above. In these 3 cases (2,4,6) a gradation appeared to exist as one moved from skin to margin to scar. Both increasing and decreasing gradations in % B isoenzyme were observed.

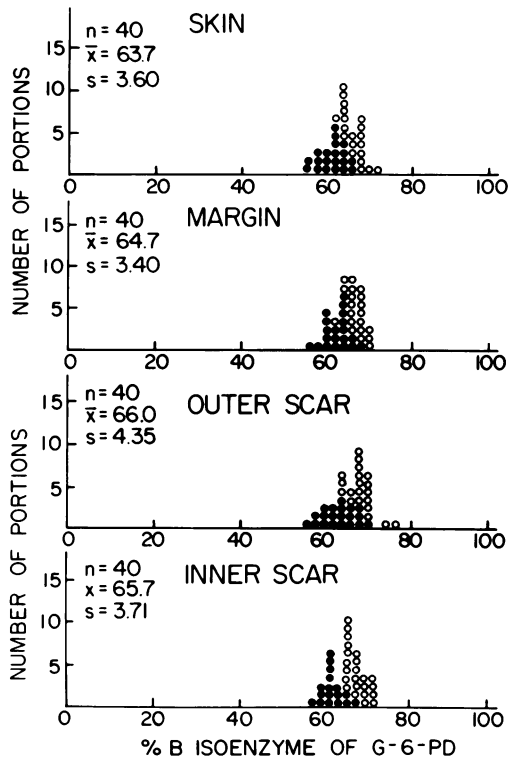

Figure 2—The isoenzyme values for portions from Case 7 are illustrated by type of portion. Each solid point represents the isoenzyme value for a lower portion; the open points represent values for upper portions of each type. The mean and standard deviation for all portions of each type are included.

However, analysis of variance showed the variance between types of portions not to be significantly greater than the variance within each type of portion. Patient age and scar age in cases that showed significant differences between isoenzyme values in portions of scar and skin were not different from those in cases without significant differences.

The mean isoenzyme values of upper and lower portions of each type of portion were also compared (Table 2). In 5 of the 7 cases, significant differences ($P < .05$) were observed between the upper and lower portions. These significant differences occured randomly in all types of portions, including adjacent skin, without any one type of portion predominating.

Individual portions containing an oligoclonal cell population (many fewer clones than normal but still more than one) could not be detected with the methods employed. However, portions taken from tissue with an oligoclonal cell population would be expected to exhibit significantly greater portion-to-portion variation. No consistent significant differences in variance were noted in any individual case between any two types of portions with the F test or between all types of portions with Bartlett's test. Thus, the variance of isoenzyme values for scar was not significantly different from the variance of values for skin or margin.

Discussion

Atherosclerosis is thought to arise from a process of smooth muscle cell proliferation.[6,7] The stimulus of this proliferation has long been the subject of debate. Virchow in 1856 first suggested that plaques arose in response to intimal injury.[8] Thus, atherogenesis has been likened to a process of intimal scarring. It might then be assumed that if the process of clonal selection occurred very frequently within plaques during the healing of this intimal injury, evidence of strong clonal selection might also be detected in cutaneous scars, particularly if the distance over which this selection might occur were comparable to the size and thickness of human atherosclerotic plaques, as it was in this study.

The healing of cutaneous wounds is carried out primarily by the proliferation of fibroblasts at and behind the margins of the healing scar and from the subcutaneous tissue.[9] Following wound closure, consolidation of the scar results in closure of capillary beds and departure of migratory cells, leaving fibroblasts, epithelium, and connective tissue. If clonal selection occurs, it should be identified within the area resulting from this hyperplastic process, showing

Table 2—Mean (± 1 SD) Values of the Percentage of B Isoenzyme of Total G-6-PD Activity for 870 Portions of 7 Human Scars by Type of Portion

		Type of portion							
		Skin		Margin		Outer scar		Inner scar	
	Upper (U) or lower (L)	N	% B G-6-PD	N	% B G-6-PD	N	% B G-6-PD	N	% B G-6-PD
Case 1	U	27	57.1 ± 10.7	23	57.0 ± 11.2	22	55.6 ± 11.5	22	55.2 ± 11.7
	L	28	49.1 ± 6.9§	26	48.1 ± 6.2§	26	50.0 ± 6.7§	22	49.5 ± 7.5
Case 2	U	10	76.5 ± 3.9	10	77.6 ± 5.0	11	78.3 ± 4.4	15	81.6 ± 3.9†‡
	L	11	76.9 ± 4.2	12	78.0 ± 4.4	11	79.0 ± 6.9	18	75.6 ± 3.9§
Case 3	U	19	61.6 ± 5.9	19	60.8 ± 4.3	18	63.3 ± 5.1	—	—
	L	19	57.2 ± 4.4§	19	57.3 ± 4.7§	18	57.8 ± 3.7§	—	—
Case 4	U	16	79.3 ±	16	76.6 ± 4.6	16	75.6 ± 3.7*	—	—
	L	16	75.5 ± 8.3	16	73.4 ± 5.6	16	71.2 ± 5.1§	—	—
Case 5	U	14	82.0 ± 3.2	14	82.5 ± 3.3	14	83.5 ± 4.1	—	—
	L	14	83.1 ± 2.4	14	82.7 ± 3.8	14	81.2 ± 3.6	—	—
Case 6	U	20	54.6 ± 2.8	20	55.2 ± 5.0	22	58.2 ± 4.9†	—	—
	L	20	53.2 ± 5.6	20	56.3 ± 4.8	22	58.9 ± 4.3‡	—	—
Case 7	U	20	61.3 ± 2.9	20	62.5 ± 3.1	20	63.2 ± 3.7*	20	62.9 ± 2.5*
	L	20	66.2 ± 2.4§	20	66.9 ± 1.9§	20	68.7 ± 3.0§*	20	68.5 ± 2.3§*
TOTAL		254		249		250		117	

* Significantly different from skin ($P < .05$).
† Significantly different from skin ($P < .01$).
‡ Significantly different from margin ($P < .05$).
§ Significantly different from corresponding upper group ($P < .05$).
‖ Scar not thick enough for inner scar portions to be dissected separately.

minimal selection at the scar's margin and maximal selection in the scar's center.

The results of this study using clonal markers indicate that only a small degree of clonal selection occurs within healing wounds. In 4 of the 7 cases, the small differences in mean values for portions of scar compared with portions of skin were nonetheless significant. In 5, an increasing trend in % B isoenzyme moving from skin to inner scar was noted; in 2, a decreasing trend was observed. Therefore, the selection was not uniformly toward the A or B isoenzyme. Furthermore, clonal selection is not the only possible explanation for the differences observed between skin and scar. Another possibility is that the scar contains cells of an origin different from the skin, and the isoenzyme differences are therefore due to differences between tissue types and not clonal selection. For example, significant differences were observed in several cases between upper and lower layers of normal skin. Thus, if the upper scar contained more cells derived from subcutaneous tissue than does normal skin, differences between upper scar and upper skin might be explained on that basis. It is also possible that the tissue was composed of larger than normal patches of cells derived from a single clone. This mechanism could produce differences in the means of samples simply due to increased sampling variation. However, this mechanism should also have produced large variances within groups of portions. These differences in variance were not observed in tests comparing variance between skin and scar.

Even if clonal selection is the mechanism responsible for the observed differences, the differences of the means are exceedingly small—5.7% being the largest observed. This is in marked contrast to the large differences observed between isoenzyme values of normal arterial wall and those of human atherosclerotic plaques from the same artery[1-3] and even between layers of the same atherosclerotic plaque and the arterial wall underlying them.[10] Furthermore, most studies of atherosclerotic plaques have been performed in small plaques whose thickness and diameter are similar to if not smaller than the thickness and width of the scars examined in this study. Finally, the laboratory methods used in this study, including the size of tissue sample used, were identical to those used in studies of atherosclerotic plaques and human tumors.[3] Thus, clonal selection may occur during wound healing, but the magnitude is not nearly enough to explain the differences observed between atherolsclerotic plaques and underlying arterial wall.

Two objections to the present study could be cited. First, it might be argued that atherosclerotic plaques

and cutaneous scars are inherently different, since they consist of different cell types (smooth muscle cells vs fibroblasts). However, there is no reason to suspect that selection in a population of fibroblasts should be markedly different from selection in a population of smooth muscle cells. Second, it might also be argued that the study of well-healed scars in which cellular proliferation is no longer occurring provides little information about the selection processes occuring in actively proliferating cells from atherosclerotic plaques. However, comparison of healed scars with actively growing plaques would seem conservative, as plaques would contain various inflammatory cells, capillaries, and other cell types that would be expected to be polyclonal. Despite the presence of these various cell types, the majority of plaques have monoclonal characteristics.[1-3] Healed scars contain a homogeneous population of fibroblasts that should favor the demonstration of monoclonality. If it can be assumed that the cells in a well-healed scar are derived from those in the more cellular, less well-healed scar that preceded it, monoclonal foci, if they were present in the healing scar, should persist as the cellularity of the scar decreases with age. We cannot altogether exclude the possibility that monoclonal cellular proliferation occurs within young healing scars. However, if that were so the absence of monoclonal features in well-healed scars could only be explained by the migration of many new cells into the scar as it becomes less cellular. Such paradoxical cellular movement into well-healed scars seems unlikely and has never been documented.

In addition to the human studies reported in this paper, we have also studied the clonal characteristics of 3- and 6-week-old skin scars in four mules. No evidence of selection was observed, but the variability of isoenzyme percentages in normal skin made the detection of minor degrees of selection impossible.

Glucose-6-phosphate dehydrogenase has been used by other investigators to study clonal selection.[11] A deviation from the ratio of A and B isoenzymes found in the tissue of origin could be evidence for a process of selection. This mechanism may explain why cells passed serially in culture tend to take on monoclonal characteristics, suggesting a selection of clones due to the tissue culture process.[12] Monoclonal characteristics have been consistently found *in vivo* in a wide variety of human neoplasms.[13,14] However, cell proliferation *in vivo* cannot alone explain the origin of monoclonal cell populations, since parathyroid hyperplasia and "adenomas" were uniformly polyclonal.[15] Thus, the finding of a monoclonal cell population *in vivo*, as in atherosclerotic plaques, would favor a mutational origin of the lesion.

The finding of minimal selection in human scars may help to explain why the findings of other investigators based on *in vitro* or animal studies are not applicable to the human atherosclerotic plaque. *In vitro* studies of fibroblasts[4] and smooth muscle cells[16] would provide one plane on which to grow, and the maintenance of cells in cultures would provide factors selective for cells with certain characteristics. Thus, *in vitro* studies provide optimal conditions in which to demonstrate selection, but their results must be interpreted with extreme caution with regard to *in vivo* phenomena. Similarly, the findings of polyclonal characteristics within microscopic lesions in the intima of hypercholesterolemic swine[17] might be due to intimal injury and could thus be expected, from the results of this study, to be polyclonal. However, we had previously shown human fatty streaks to have polyclonal characteristics,[2,3] and thus these small cholesterol-induced experimental lesions may resemble the fatty streak more than the fibrous plaque.

In summary, this study provides evidence that clonal selection may occur during the healing of cutaneous wounds. However, the extent of the selection is extremely small, with only a 5.7% maximum change in the percentage of B isoenzyme content. It is thus concluded that clonal selection during repair of arterial injury is an unlikely mechanism to explain monoclonality within human atherosclerotic plaques.

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