Acute Inflammation Loci Are Involved in Wound Healing in the Mouse Ear Punch Model

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Significance: Molecular biology techniques are being used to aid in determining the mechanisms responsible for tissue repair without scar formation. Wound healing is genetically determined, but there have been few studies that examine the genes responsible for tissue regeneration in mammals. Research using genetic mapping is extremely important for understanding the molecular mechanisms involved in the different phases of tissue regeneration. This process is complex, but an early inflammatory phase appears to influence lesion closure, and the present study demonstrates that acute inflammation loci influence tissue regeneration in mice in a positive manner.

Recent Advances: Mapping studies of quantitative trait loci (QTL) have been undertaken in recent years to examine candidate genes that participate in the regeneration phenotype. Our laboratory has identified inflammation modifier QTL for wound healing. Mouse lines selected for the maximum (AIRmax) or minimum (AIRmin) acute inflammatory reactivity (AIR) have been used to study not only the tissue repair but also the impact of the genetic control of inflammation on susceptibility to autoimmune, neoplastic, and infectious diseases. Murphy Roths Large and AIRmax mice are exclusive in their complete epimorphic regeneration, although middle-aged inbred mice may also be capable of healing.

Critical Issues: Inflammatory reactions have traditionally been described in the literature as negative factors in the process of skin injury closure. Inflammation is exacerbated due to the early release of mediators or the intense release of factors that cause cell proliferation after injury. The initial release of these factors as well as the clean-up of the lesion microenvironment are both crucial for following events. In addition, the activation and repression of some genes related to the regeneration phenotype may modulate lesion closure, demonstrating the significance of genetic studies to better understand the mechanisms involved in the initiation of wound repair processes.

Future Directions: The pleiotropic effects of the QTL are important in the identification of the genes responsible for tissue repair processes, especially when combined with global gene expression research. Microarray analysis complements the biological information obtained in QTL mapping, making this tool essential for gene identification. This approach will allow the investigation of future targets for therapeutic wound healing treatments.

SCOPE AND SIGNIFICANCE

TISSUE REPAIR is a complex biological process that involves a series of sequential events within the immune system after injury.¹ Skin in-

jury responses involve two distinct processes: healing and regeneration.^{2,3} Regeneration is rarely observed in mammals, but this process is largely determined by an intense

but short-duration inflammatory event after injury. Maximum acute inflammatory reactivity (AIRmax) mice genetically selected for acute inflammation demonstrated fast ear hole closing, with regeneration comparable to that observed in both Murphy Roths Large (MRL) and LG/J mice,^{4,5} which are inbred mice lines that demonstrate epimorphic regeneration.⁶ Epimorphic regeneration (epimorphosis) is a type of regeneration that involves dedifferentiation of adult structures to form undifferentiated masses of cells. These cells proliferate rapidly and accumulate under the epidermis, which has already expanded. These dedifferentiated cells and their epidermal covering are called regeneration buds or regeneration blastema. The dedifferentiated cells continue to proliferate and finally redifferentiate to form a rudiment tissue that eventually transforms into a specific organ, such as limb regeneration in amphibians or mouse ear punch healing. The regeneration capacity of these mice is due, in part, to quantitative genetic control.⁷ Genetic studies were performed using the AIRmax and minimum acute inflammatory reactivity (AIRmin) mice to determine the relationship of inflammatory loci to the regenerative wound healing phenotype, and showed that the variability of this phenotype is highly influenced by the interaction of acute inflammation quantitative trait loci (QTL) located on chromosomes 1 and 14,⁴ as well as on chromosomes 7, 8, 12, and 16 (unpublished data*)—which suggests the existence of shared genetic mechanisms in inflammation and regeneration processes.

TRANSLATIONAL RELEVANCE

Uncovering the genetic interactions of inflammation and tissue repair in animal models will allow the establishment of experimental human protocols to safely investigate therapeutic agents and processes that can facilitate damaged tissue regeneration. Many studies using mice and rabbits are currently being undertaken to identify candidate genes and biochemical pathways that could be future sources of new medicines and treatments.

CLINICAL RELEVANCE

Researchers in different parts of the world are currently committed to investigating the mecha-

nisms responsible for the complete renewal of damaged tissues. As tissue repair processes are known to be genetically controlled,⁸ one of the most important lines of treatment for skin diseases, and even accidents such as burns, will be gene therapy—but how these processes can be manipulated for therapeutic intervention is still an important issue to be examined in mammalian tissue repair models.

EXPERIMENTAL MODEL OR MATERIALS: ADVANTAGES AND LIMITATIONS

Mice that were genetically selected for AIRmax or AIRmin in our laboratory have been used to study the genetic control of nonspecific immunity in autoimmune disease susceptibility,⁹ neoplasia,¹⁰ and infectious diseases.¹¹ These two strains were obtained through bidirectional genetic selection, starting with a genetically heterogeneous founder population (F0) produced by intercrossing eight isogenic strains of mice of independent origins (A/J, DBA/2J, P/J, SWR/J, SJL/J, CBA/J, BALB/cJ, and C57BL/6J; Fig. 1). The phenotypes chosen for selection were the local leukocyte influx and exudated plasma proteins 24 h after the subcutaneous injection of polyacrylamide beads (Biogel), a nonantigenic, insoluble, and chemically inert substance.¹² The progressive divergence of the AIRmax and AIRmin lines in leukocyte infiltration and exudated protein concentrations after the selection process reached 20- and 2.5-fold, respectively. These differences resulted from the accumulation of alleles endowed with opposite and additive effects on the inflammatory response. AIRmax and AIRmin mice were therefore developed from eight inbred lines for strong and weak acute inflammation phenotypes, are homozygotic for acute inflammation modifier loci, but have heterogeneous backgrounds as inbreeding intercrosses were avoided during the selective process. Genetic studies during the selective processes indicated that the acute inflammation phenotype involves at least 11 QTL.¹⁰ The inflammatory response to Biogel, as well as susceptibility to pristane-induced arthritis,¹³ *Salmonella enterica* serotype Typhimurium infection,¹¹ and bacterial lipopolysaccharides (LPSs)¹⁴ were all modified in these mice. Additionally, linkage analyses using microsatellite markers suggested that the presence of QTL in chromosomes 1, 6, and 11 was relevant to these phenotypes.¹⁴ Alterations in bone marrow granulopoiesis in response to hematopoietic factors and the production of chemotactic factors by infiltrated or local resident cells both contribute to

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Figure 1. Scheme used for the production of the foundation population (F0) by the intercrossing of eight inbred strains of mice, containing the *Slc11a1* alleles of each strain. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

phenotypic differences between the two lines. Convergent phenotypes in AIRmax mice were observed that were characterized by high neutrophil production in bone marrow, a high number of neutrophils in the blood, high concentrations of chemotactic agents, and increased resistance of infiltrating neutrophils to spontaneous apoptosis.¹⁵ Inflammation resolution seems to have been affected by the acute inflammation selective process, as observed during lung carcinogenesis induction using urethane. Persistent subacute inflammatory reactions in the lung parenchyma, with granulocyte and lympho-mononuclear cell infiltration, were observed 21 days after urethane treatment in AIRmin mice, while these responses were reduced in AIRmax mice, indicating that they had higher inflammation resolutions.¹⁶ The AIRmax and AIRmin mice produced in our laboratory show the genetic heterogeneity necessary for studies of natural populations and can therefore be used as experimental models in studies that examine human wound healing.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

The development of experimental approaches appropriate to investigating multifactorial genetic characters, such as acute inflammatory reactions,

has been the main objective of our research. These models consist of accumulations of allelic variations through bidirectional genetic selection that are determinant of phenotypic expression in extreme strains of mice. An important result has recently been obtained in the detection of several loci that control the intensity of acute inflammation through analyses of single-nucleotide polymorphisms (SNPs). Six inflammatory QTL involved in the tissue regeneration phenotype in AIRmax and AIRmin mice were found to be located on chromosomes 1, 7, 8, 12, 14, and 16. These loci harbor several candidate genes involved in tissue regeneration and in determining sensitivity to experimentally induced diseases. The *solute carrier 11a1* gene (*Slc11a1*) on chromosome 1 is the most likely candidate, and its participation in the early events of inflammatory ear tissue regeneration was observed.⁵ The *Slc11a1* gene has been shown to be highly pleiotropic as it acts on the transport of iron, zinc, and manganese ions in phagocytic cells, interfering with macrophage activation; oxidative and nitrosamine bursts¹⁷; TNF- α , IFN- γ , and IL-1 production¹⁸; and the expression of MHC class II molecules.¹⁹ A functional mutation on codon 169 of the *Slc11a1* gene that determines susceptibility to several infectious diseases resulted in the Susceptible (SS) and Resistant-wild-type (RR) alleles. The frequency of the *Slc11a1* SS allele was 25% in

the initial population (F0) of the AIR selection experiment but shifted to 60% in AIRmin and 9% in AIRmax after 30 generations of selective breeding. This frequency change appears to be the result of selection processes involving this gene (or other closely linked genes) in the control of the acute inflammatory reaction intensity.¹¹ The *Slc11a1* gene maps to 38.54 cM in chromosome 1. *Slc11a1* homozygous AIR sublines were produced by genotyping heterogeneous AIRmax and AIRmin mice with specific PCR primers for the *Slc11a1* SNP functional mutation at the 169 codon. Homozygous or heterozygous mice were mated in each line to obtain AIRmax and AIRmin mice presenting *Slc11a1* *R* and *S* alleles in homozygosity while maintaining their heterogeneous genetic background. After the production of AIRmax^{RR}, AIRmax^{SS}, AIRmin^{RR}, and AIRmin^{SS} lines by genotype-assisted breeding, several polymorphic microsatellites near this gene were analyzed to determine the haplotype of this chromosome 1 region. The microsatellite allele segregation patterns of the different lines were independent of the fixation of the *Slc11a1* *R* or *S* allele but were similar to those found in the parental AIRmax and AIRmin mice. The minimal intervals that encompass the *Slc11a1* gene region were ~4 cM in all lines, and *Il8rb* is located in the same region. However, no microsatellite polymorphisms between the lines could be observed for the *Il8rb*

gene.¹³ Acute inflammation to Biogel, and susceptibility to *S. Typhimurium* infection and LPS were modified in these lines, and inflammatory QTL were found in chromosomes 1, 6, 11, and 13.¹⁴ Studies with these lines demonstrated that AIRmax^{SS} mice had potent ear hole regeneration capacities comparable to the isogenic MRL strain. AIRmax^{RR} mice demonstrated mild regeneration, suggesting the involvement of the *Slc11a1* gene in the genetic control of the regeneration phenotype. AIRmin^{RR} and AIRmin^{SS} mice did not regenerate ear holes (Fig. 2).⁴ These results suggest that fine tuning of inflammatory intensity is decisive for complete regeneration. The first global gene expression study using this ear punch wound healing mouse model investigated the inflammatory gene pathways in MRL and B6 mice and suggested that fast wound repair in MRL mice was mediated by a metabolic shift to a low inflammatory response.²⁰ Few genes related to cell proliferation were different between both lines at 2–3 weeks after ear punch, indicating an important role of the early inflammatory events. In contrast, our global gene expression analyses showed the early activation of genes involved in inflammatory responses (48 h after injury) and the suppression of genes related to muscle contraction in AIRmax^{SS} mice and complete regeneration. Low *Actn3*, *Acta1*, *Smtnl1*, *Myh11*, *Myl9*, and *Mybpc2* gene expression was observed and was associated with fibroblast dif-

Figure 2. Ear hole closure. A 2-mm through-and-through ear hole was made and the same wound was followed for 30 days. From top to bottom, the hole closure was photographed on days 1 and 30. AIRmax^{SS} mice showed fast ear tissue regeneration while AIRmin^{SS} mice did not show regeneration after ear punch. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

ferentiation into contractile myofibroblasts that play an important role in injury resolution and cell matrix deposition, contributing to scar formation in the repair process rather than to a regenerative process.^{21,22} As these genes are downregulated in AIRmax^{SS}, scar formation can be decreased in these mice. The involvement of inflammatory cells in wound healing has been widely investigated,^{23,24} and there is evidence for both positive and negative controls. Many studies have suggested that neutrophils inhibit wound repair processes. Neutrophil-depleted mice showed epidermal healing that was significantly faster than that observed in control mice.^{25,26} Inflammatory responses mediated by neutrophil and macrophages correlate with increased matrix metalloproteinase activity, blastema formation, and regeneration in the MRL mouse ear-hole model,²⁷ although vigorous inflammation can delay wound closure. The involvement of activated inflammatory genes at the beginning of this process contradicted some studies that have reported a negative influence of intense inflammatory events on regeneration, with prolonged inflammation inducing tissue fibrosis. However, AIRmax^{SS} mice seem to quickly resolve acute inflammation, favoring repair processes.⁴ AIRmax and AIRmin mice differed 20-fold in the number of infiltrated leukocytes and 2.5-fold in protein concentration in the 24-h subcutaneous inflammatory exudate. These differences in cell infiltrate reflect alterations in bone marrow granulopoiesis in response to hematopoietic factors, as well as to the ability of AIRmax exudate granulocytes to resist apoptosis. The high local production of chemotactic factors by infiltrated or local resident AIRmax cells also contributes to the phenotypic difference between the lines. AIRmax mice also display high susceptibility to arthritis induction,⁹ as do MRL and DBA/1 inbred mice,^{28,29} which are also good wound healers.^{6,30} As in arthritis, acute-phase proteins and chronic inflammatory events seem to be involved in wound healing.^{31,32} We have also demonstrated that fast-regenerative AIRmax^{SS} mice are extremely susceptible to pristane-induced arthritis, showing the high levels of cytokines involved in chronic inflammation.¹³ Further, the different abilities of AIRmax and AIRmin mice to induce an initial polymorphonuclear cell influx and to repair damaged tissue might both in part explain their different susceptibility to lung tumor development after urethane treatment. Histological analyses showed that AIRmin mice do not control lung inflammatory process and developed cancer.¹⁶ Lung of untreated AIRmax or AIRmin mouse showed a

specific gene expression profile associated with modulation of inflammatory response.³³ The genes involved with the leukocyte transendothelial migration, cell adhesion molecules, and tight junction pathways were observed in AIRmax lung and might be relevant to explain the control of the inflammatory response in these animals.³³ In addition to the importance of regulating inflammation during initial healing events, activation and repression of some genes related to the wound healing phenotype can modulate tissue repair. There are many candidate genes in the loci detected (as shown in Fig. 3) in the AIRmax higher regenerative line in regards to inflammation (Fig. 3A), signal transduction molecules (Fig. 3B), and on chromosome 1 (Fig. 3C). Differentially expressed genes between AIRmax and AIRmin in the detected loci were presented in Figure 4. Interestingly, a cluster of keratin genes was observed on chromosome 16 and genes related to inflammation, leukocyte migration, and cell adhesion were verified on the other loci. Additional genetic studies will need to be undertaken to narrow the confidence intervals of these loci to better gene candidates. These results provide an interesting evidence of cellular and molecular early inflammatory mechanisms involved in tissue restoration, which could also exert influence on other experimental diseases, such as infections,^{11,14,34} arthritis,^{9,13} envenoms,^{35,36} and cancer susceptibility.^{37,38} Further studies should be carried through to a deep understanding of the genetic and cellular mechanisms involved in the several phases of tissue regeneration, in which our lines of mice certainly will contribute to identify genes that modulate the inflammatory phase of the epimorphic regeneration.

INNOVATION

Several genetic mapping studies are currently being developed using inbred mice. A number of crosses were made between "regenerative" and "nonregenerative" strains to identify potential candidate genes that show phenotypic effects. Most genetic mapping studies have used MRL and LG/J regenerating lines crossed with resistant strains, such as C57BL/6, DBA1, SM, and CAST. Approximately 20 loci were detected, some of which were found in the same regions of AIRmax and AIRmin mice, suggesting common genetic elements among those models. The combined cross analysis,³⁹ fine mapping, and production of congenic animals⁴⁰ are interesting approaches to the accurate identification of the genes responsible for regeneration capacity phenotypes at these loci. While the use of

Figure 3. Up- and downmodulated genes in Airmax mice in relation to inflammation (A), signal transduction molecules (B), and on chromosome 1 (C). Total mRNA was isolated from mouse ear tissues in order to compare with control mice on Mouse Gene 1.0 ST Array (Affymetrix—28 kb genes) to identify sets of gene differentially expressed. Significance of Analysis of Microarray analysis using two class unpaired, $FDR \leq 5\%$ ($p < 0.001$), revealed distinct gene expression profile between Airmax control and Airmax submitted to wound healing. Global gene expression analyses were performed 24 h after hole punch that examined the data from four biological replicates on each line. FDR, false discovery rate; mRNA, messenger RNA. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

Figure 4. Differentially expressed genes between AIRmax and AIRmin in the six loci detected. Total mRNA was isolated from mouse ear tissues in order to compare with control mice on Mouse Gene 1.0 ST Array (Affymetrix—28 kb genes) to identify sets of gene differentially expressed. Significance of Analysis of Microarray analysis using two class unpaired, FDR $\leq 5\%$ ($p < 0.001$), revealed distinct gene expression profile between AIRmax and AIRmin mice submitted to wound healing. Global gene expression analyses were performed 24 h after hole punch that examined the data from four biological replicates on each line. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

these strains is important, the homogeneity of inbred mice lines restrains the biological significance of the results. The use of lines that have been genetically selected for high and low inflammatory responses from outbred mouse stocks is an innovative approach to analyzing the mechanisms involved in regeneration phenotypes as these strains have heterogeneous backgrounds representing extreme phenotypes similar to those found in natural populations. This study allowed us to demonstrate that when inflammation is genetically regulated during its early stages it has a fundamental role in the healing outcome.

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS

The low numbers of known genes that control features of interest, the high costs of the initial stages of experiments, and the pleiotropic effects of other genes on candidate genes create difficulties in using genetic mapping. Although the variability to establish causal gene responsible for its quantitative effect, there is always the possibility that the focal gene is not actually the one causing the observed difference in a given characteristic, but that it is only linked to the QTL. This can lead to conflicting results depending on the population studied, because significant associations in a specific population may not be observed in others. Another constraint is that the genome region identified may be relatively large and contain many genes, making it difficult to identify the specific genes that explain the QTL.

FUTURE DEVELOPMENTS OF INTEREST

Combinations of various molecular biology techniques have allowed us to advance in our basic research of wound repair. Several studies have identified different regions in the mouse genome responsible for lesion closure.^{39–44} We intend to carry out a fine mapping of the QTL that regulate

TAKE-HOME MESSAGES

Basic science advances

Global tracking of the mouse genome and inserting or deleting genes of interest in studies of the mechanisms that modulate a particular phenotype are very promising techniques. Genomic studies with experimental models represent an important approach to identifying and developing biomarkers of healing.

Clinical science advances

The availability of techniques with high therapeutic potential, including studies using microRNAs. These noncoding regulatory RNAs are now widely used in treating patients with fibrosis or slow healing wounds.

Relevance to clinical care

Identification of candidate genes that participate in the healing phenotype may contribute to the regulation of gene expression related to the process, such as the inflammatory response in the early healing. The modulation of the initial inflammatory effects involves additional mechanisms, such as the deposition of an organized and controlled extracellular matrix, providing better quality healing.

the intensity of inflammatory responses to high-density SNPs in future research projects to reduce the confidence intervals and to address differences in global gene expression protocols and correlate them with the QTL in order to implicate fewer genes in these loci. Further experiments that investigate the involvement of microRNAs (which regulate the expression of many genes) as well as the loss of functions of these RNA sequences may focus additional emphasis on the wide range of potential clinical applications of this technology.

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AUTHOR DISCLOSURE AND GHOSTWRITING

The authors have no conflicts of interest to disclose. The listed authors were solely responsible for writing this article.

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Abbreviations and Acronyms

Acta1 = actin, alpha 1, skeletal muscle

Actn3 = actinin alpha 3

AI Rmax = mice genetically selected for maximal acute inflammatory reactivity

AI Rmax^{RR} = mice genetically selected for maximal acute inflammatory reactivity homozygous for *Slc11a1* R alleles

AI Rmax^{SS} = mice genetically selected for maximal acute inflammatory reactivity homozygous for *Slc11a1* S alleles

AI Rmin = mice genetically selected for minimal acute inflammatory reactivity

AI Rmin^{RR} = mice genetically selected for minimal acute inflammatory reactivity homozygous for *Slc11a1* R alleles

AI Rmin^{SS} = mice genetically selected for minimal acute inflammatory reactivity homozygous for *Slc11a1* S alleles

cM = centimorgan

FDR = false discovery rate

IFN- γ = interferon gamma

IL-1 = interleukin 1

IL8rb = interleukin-8 receptor type beta

LPS = lipopolysaccharide

MHC II = major histocompatibility complex-class II

MRL = Murphy Roths Large

mRNA = messenger RNA

Mybpc2 = myosin binding protein C, fast-type

Myh11 = myosin, heavy polypeptide 11, smooth muscle

MyI9 = myosin, light polypeptide 9, regulatory

QTL = quantitative trait loci

Slc11a1 = solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1

Smtnl1 = smoothelin-like 1

SNPs = single-nucleotide polymorphisms

TNF- α = tumor necrosis factor alpha