The phenotype in complex diseases is profiled by/for epigenetic factors

Recent advances in knowledge of the mechanisms regulating gene expression have changed our conception of complex diseases such as cancer, lupus, and rheumatoid arthritis (RA). These polygenic traits are associated with an array of low-penetrance genes that confer susceptibility or protection and are transmitted via a non-mendelian inheritance pattern. Environmental factors that may participate in the development of RA are largely unknown, which makes it more difficult to predict its appearance. Patients who seek medical care most likely represent only a small segment of the arthritic population. It would also be of great interest, however, to study the patients...
who have a mild and remitting disease course to identify the factors that determine its progression. In this regard, complex diseases are characterised by a wide spectrum of severity that reflects the cumulative effect of various permissive factors.

Regardless of the genetic load, transcription activity in the genes is exquisitely regulated for each specific tissue in relation to the evolutionary status. During embryonic development, a repression takes place in the expression of a great number of genes; this progresses steadily throughout cell divisions and is responsible for specification. This is effected through a program of epigenetic silencing—a term that was coined to highlight the stable suppression of gene expression that occurs whereby transmittable patterns are created in the daughter cells without involving changes in the germ line. Although the full extent of epigenetic modifications is not yet understood, it is already evident that basic processes such as cell growth, hormone secretion, inflammatory response and immune cell maturation are governed by this type of regulation. Likewise, degenerative diseases, inflammation, and cancer are characterised by an accumulation of epigenetic marks in diseased cells. This has given rise to the hypothesis that various environmental factors, past and recent, bring about alterations in the patterns of gene expression through epigenetic mechanisms and that these mechanisms could explain to a great extent how the environment contributes to the development of complex diseases.

**Epigenetic mechanisms in transcriptional silencing**

Gene transcription is inhibited through DNA methylation and the changes in chromatin conformation. These two mechanisms act in coordination to make the genes inaccessible for reading (Figure 1). The chromatin is stabilised by covalent bonds between the DNA and the histones and other basic proteins of the cell nucleus. Posttranslational modifications in the histones trigger changes in their affinity for bonding with DNA, generating a closed or open chromatin conformation (hetero- or euchromatin, respectively). Both DNA methylation and histone modifications are reversible processes, catalysed by specific enzymes and cofactors. This indicates that cells are capable of changing their epigenetic expression patterns in response to various stimuli, which, at the same time, allows them to be manipulated for a therapeutic objective. Many of these epigenetic marks will however have to await the next mitotic division, when they can be modified.

Methylation of the DNA occurs through the action of DNA methyltransferases (DNMT) in certain regions rich in cytosine phosphoguanine dinucleotides. For their part, H3 and H4 histones can undergo numerous transformations such as phosphorylation, methylation, acetylation, ribosylation, sumoylation, and ubiquitination, as well as the inverse reaction and various combinations of reactions. Most of these take place in the amino acids lysine (K) or arginine (D) near the amino-terminus end of the protein. In the long list of enzymes that participate in histone modifications, the histone-acetylase (HAT) and histone-deacetylase (HDAC) families are prominent. The elimination of acetyl groups is closely related to transcriptional silencing, while acetylation opens the chromatin conformation and is associated with increased gene expression (Figure 1).

**Epigenetic mechanisms in rheumatoid arthritis**

The inflammatory response in septicaemia and in a flare-up of RA show initial cytokine bursts that are very similar; in septicaemia, however, this wave gives way to a drop in cytokine levels that does not occur in arthritis. This indicates that, in arthritis patients, there is a defective control in ending the process. The primary route leading to production of proinflammatory cytokines, both during sepsis and in RA, is the innate activation pathway for transcription factor (NF) κB. This is a clear example of how the epigenetic mechanisms intervene in the regulation of cellular responses. Factor NFκB is found in a latent state in cell cytoplasm, anchored to its inhibitor, IκB. In turn, the inhibitor’s availability is regulated through methylation of its promoter—a situation that can result in significant differences in cytokine production. In the regions corresponding to the proinflammatory cytokine promoters, the chromatin is also folded into a closed conformation. In synchrony with the translocation of NFκB to the nucleus, transitory changes in this conformation must occur (a demethylation in K9 and a phosphorylation in serine 10 (S10) of the H3 histone) to allow the transcription machinery to be incorporated into its bonding sites on the promoters for tumour necrosis factor alpha (TNFα) and interleukin 1β (IL-1β).

Another of the processes closely regulated by epigenetic mechanisms is the differentiation of immunocompetent cells. Rheumatoid arthritis is characterised by polarisation toward Th1 and Th17 responses. Differentiation of the double-positive T cells toward Th1 responses is brought about through progressive methylation of the promoter for IL-4, which is the cytokine specific for Th2 cells, until it has been completely suppressed. For its part, transcription factor FoxP3, which defines regulatory T cell functions, is susceptible to silencing by methylation—an event that skews the balance toward production of Th17 cells and favours autoimmunity. These changes in T cell phenotype are largely reversible, and there is evidence that some cytokines such as transforming growth factor beta (TGFβ), IL-6, and interferon gamma (IFNγ) actively participate in establishing epigenetic patterns in cell subpopulations.

**The invasive phenotype**

The earliest information about epigenetic involvement in human pathology came from tumours, where there was evidence that the presence of certain epigenetic marks promoted the invasiveness
and survival of the affected cells. In particular, an event typical of
the initial phases of tumorigenesis is the de novo methylation of
tumour regulatory or suppressor genes.\textsuperscript{15} In this regard, although
DNMTs are essential to obtain normal patterns of gene methylation,
their inhibitors have been revealed as an interesting strategy for
making cells re-express abnormally hypermethylated genes.\textsuperscript{26} The
first demethylating agents employed in the treatment of human
disease were 5-azacytidine and 5-aza-2’-deoxycytidine (5-aza-CR
and 5-aza-CdR), followed by the less toxic Zebularine and MG98. The
role of HDACs in the gene silencing associated with dedifferentiation
of some tumours and with their resistance to chemotherapy has
also been ascertained. Suberoylanilide hydroxamic acid (SAHA) is
a natural inhibitor of HDAC activity that is approved for treatment
of cutaneous T cell leukaemia, while 2 other antagonists of these
enzymes, phenylbutyric acid and valproic acid, have demonstrated
their ability to correct altered gene expression patterns in various
diseases.\textsuperscript{27}

The degree of genome methylation in the rheumatoid synovial
membrane is being studied because of the similarity between
the local fibroblasts and the transformed cells. Although they lack
mutations, the synovial fibroblasts (SF) in these patients show an
abnormally elevated survival and execute an invasive program
characterised by protease and cytokine secretion.\textsuperscript{22,23} In the joints
of these patients, the cells found in areas where bone and articular
cartilage have been invaded express proto-oncogenes and growth
factors.\textsuperscript{24} This local-regional pattern of gene expression suggests
that, beginning with the inflammatory environment, a particular
epigenome could be triggered, propagating to the progeny of the
modified cells. Aspects of these mechanisms are beginning to be
deciphered. For example, the characteristic longevity of SF in RA
has been related to a high degree of methylation in the promoter
for proapoptotic molecules such as death receptor 3 (DR3).\textsuperscript{25} Likewise, it
has been demonstrated that trichostatin A (TSA), an HDAC inhibitor,
induces the re-expression of other proapoptotic molecules in these
cells, which results in their increased senescence.\textsuperscript{26,27} These findings
suggest that the synovial cells in RA have an altered gene silencing
program that could be related to their destructive tendency and is
amenable to reprogramming.

Pros and cons of epigenetic therapies and their possible use in
rheumatoid arthritis

Although the safety profile for epigenetic treatments in cancer
patients is still pending confirmation, we can predict that these
treatments will be introduced for non-neoplastic diseases such as RA
in the near future.\textsuperscript{28} The primary goal of epigenetic approaches in RA
will be to increase the expression of regulatory genes that have been
abnormally silenced, in the hope that this will attenuate the clinical
manifestation of the disease. This can be achieved either by inhibiting
DNA methylation or by changing the chromatin conformation in
certain places. The primary counterpart involved in using these
strategies is the risk of eliminating constituent epigenetic marks
(such as those that prevent the expression of transgenes) in cells
with a high replication index.\textsuperscript{19}

Demethylating agents

These are, by far, the epigenetic drugs that are most promising
for the treatment of RA. It is plausible that the de novo methylation
of certain regulatory genes favours RA progression and that their re-
expression would lead to a milder phenotype or improve the response
to disease-modifying drugs (Figure 2A). In reality, some of the drugs
currently used in the treatment of arthritis could act as demethylating
agents. In the earliest studies of epigenetic modifications in cancer,
epigenetic effects were observed in the majority of antineoplastic
agents when these were administered at non-cytotoxic doses.\textsuperscript{4,20}
Methotrexate, as a dihydrofolate reductase-antagonist, limits the
action of DNMTs (Figure 2B). Folic acid acts as acceptor for methionine
groups from homocysteine in the presence of vitamin B12. Methionine,
in turn, is the donor for the methyl groups that the DNMTs use in
methylation of the cytosine residues in DNA.\textsuperscript{30} This suggests that
methotrexate hinders the de novo methylation reactions (Figure
2B)—an action that could account for part of its beneficial effect in RA.
Given that IL-6 participates in genome methylation processes and in
interactions with the HDACs,\textsuperscript{21} it is also plausible that therapeutically
inhibiting it through the use of tocilizumab would have an impact
on the changes in epigenetic patterns associated with the disease
and that, in future, these changes could be associated with clinical
response groups. There is a concern that the use of demethylating
agents could result in increased levels of metalloproteinases 3 and

\textbf{Figure 2.} Actions of the DNA methyltransferase inhibitors. A) Prediction of the effects
of treatment with demethylating agents in rheumatoid arthritis. As the studies
available indicate, using these treatments would have a beneficial impact on the
primary pathogenic processes in rheumatoid arthritis: synovitis, T cell polarisation,
and innate immunity. B) Effect of methotrexate (MTX) on the action of the DNA
methyltransferases (DNMTs). DHF indicates dihydrofolate acid; SAH, sulfo-adenosyl
homocysteine; SAM, sulfo-adenosyl-methionine; THF, tetrahydrofolic acid.
13–molecules implicated in articular destruction. These molecules have cytosine phosphoguanine dinucleotides in their promoter, susceptible to methylation. In SF cultures, metalloproteinase 3 production increases following exposure to 5-aza-CdR, while the increased expression of both proteases in osteoarthritic cartilage has been related to the low degree of methylation of their promoter in diseased chondrocytes compared to healthy chondrocytes. Even so, everything appears to indicate that the threshold required to eliminate constituent epigenetic marks is higher than that required to hinder de novo methylation.

**Histone deacetylase inhibitors**

Although there are high expectations surrounding the use of HDAC inhibitors in the treatment of RA, these compounds have yet to demonstrate that they are viable candidates. Efficacy in several experimental models similar to RA has been shown by FK228, where it was capable of reducing articular inflammation and the destructive tendency. This beneficial impact has been attributed to an antiangiogenic effect and to the induction of proapoptotic molecules in the synovial tissue. In addition, beneficial effects from the HDAC inhibitors SAHA and MS-275 have been observed in rats with collagen-induced arthritis. It is difficult to translate these effects to human disease, however, for various studies have failed to demonstrate elevated HDAC activity in rheumatoid synovial tissue. On the contrary, it appears that there is an increased HAT/HDAC ratio that, in turn, is related to production of proinflammatory molecules; this fact makes us cautious when contemplating the HDAC-inhibitor approach to the disease.

**Histone methyltransferases**

The therapeutic potential of the histone methyltransferases has not been explored very much so far, even though it is well established that lysine demethylases LSD1 and Jumonji participate in the progression of various tumours. This is a very broad field in which each enzyme shows a high degree of selectivity for modifying particular residues. The family of Jumonji lysine demethylase increases its expression under hypoxic conditions, which is why they are assumed to be involved in the inflammatory responses arising from cellular stress. In particular, the methylation status of residues H3K4, H3K9, and H3K27 regulates NFκB transcriptional activity, which can be crucial in treatment design.

**Combination therapies**

The events in the cell nucleus take place in coordination and that the posttranslational modifications affect not only the histones but also the transcription cofactors and activators. It therefore seems reasonable to use epigenetic treatments in a cocktail. This strategy has shown a beneficial synergism in tumour management and has improved the safety profile as well, because it allows for therapeutic doses to be reduced. Valproic acid is a short-chain fatty acid that acts as an inhibitor to various HDACs. Retinoic acid is capable of sequestering and releasing transcription factors in the cell nucleus, displacing the HDAC-formed complexes The combination of these two drugs with 5-aza-CdR achieved re-expression of the silenced regulatory genes in tumour cells, and it has already been used in patients with acute myeloid leukaemia and high-risk myelodysplastic syndrome. Another example of synergism is the combination of 5-aza-CdR with TSA, the non-selective HDAC inhibitor, resulting in the re-expression of oestrogen receptors in a breast cancer line, which lead to a sensitization to the action of tamoxifen. It is obvious that exporting these treatments to non-neoplastic pathologies such as RA would require a high level of safety; it seems plausible, however, that combinations similar to those mentioned could be prepared, after selecting the altered processes in patients with RA on an individualised basis.

**Epilogue: treatment challenges in rheumatoid arthritis**

The main lesson learnt from the so-called biological treatments is that RA is a heterogeneous entity requiring an individualised therapeutic approach. In applying the best treatment to each case, the challenge is to categorise patients by their risk of progression and their probability of response. Just as we make use of genetic tools such as HLA and DR haplotypes and the large-scale study of polymorphisms (HapMap), in the immediate future, our knowledge of RA epigenetics should be transformed into tools of predictive and prognostic value and they should be integrated with those already available. In simplified terms, some of the measurements could be determination of the HAT/HDAC ratio and the degree of DNA methylation in appropriate patient specimens. At the present time, work is already underway to obtain these patterns on a large scale, as well as to map the microRNA system (MirMap)—not mentioned in this review but of equal importance to comprehending the pathogenesis of autoimmune diseases. Knowledge of the epigenetic mechanisms is providing us with a way to improve our understanding and use of the existing treatments for rheumatoid arthritis. Moreover, some pathogenetic circuits appear to be ideal candidates for reprogramming in the patients’ synovial fibroblasts and mononuclear cells. For the time being, in using epigenetic agents in RA, their selectivity and their uptake by the target tissue must be optimised to prevent the appearance of undesirable effects. However, analogous to what has been observed with biological treatments, the success of epigenetic therapies will depend upon the proper selection of patients.

**Conflict of interest**

The authors declare that they have no conflicts of interest.

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