MINIREVIEW

Oxygen-Reactive Species and Antioxidant Responses during Development: The Metabolic Paradox of Cellular Differentiation

(43171A)

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Abstract. **Metabolic gradients are established during early phases of development and their existence influences subsequent developmental events. Variations in oxygen supply and oxygen metabolism associated with the gradation of metabolic rate in embryos appear to form one basis for the influence of metabolic gradients on development. The rate of oxygen metabolism affects the rate of oxidant generation by various cellular biochemical pathways. Cells contain antioxidant defenses that respond to variations in cellular oxidant production. Large changes in the activity of the antioxidant enzyme superoxide dismutase and changes in cellular redox state occur during the differentiation of many types of cells. These changes correspond to an increased rate of oxidant production; the cellular environment becomes more prooxidizing during differentiation. Evidence is presented that implicates oxidants as a factor that can stimulate alterations in gene expression. Possible mechanisms by which oxidants influence gene expression are also discussed.** [P.S.E.B.M. 1991, Vol 196]

Development results from highly coordinated changes in gene expression that alter both the morphologic and physiologic characteristics of cells. The appearance of new tissues is preceded by the transcription of tissue-specific genes and the concomitant suspension of transcription of genes that are specific to pleuripotent stem cells. The nature of the mechanisms that repress or derepress the expression of specific genes at critical junctures of developmental programs remain obscure. It is known that early expression of homeotic genes regulates the expression of other genes during subsequent developmental phases; however, the factors that influence the timing of these effects and that permit the differential expression of the homeotic genes themselves are unknown.

Epigenetic factors appear to stimulate some of the development-associated changes in gene expression. The environmental forces which influence development are not distributed evenly, but instead occur as gradients that differentially alter the microenvironment of cells in different regions of an embryo. Even at the single-cell stage, gradients consisting of differentially distributed molecules in a common cytosol have been postulated to influence the expression of regulatory developmental genes (1). Although early developmental biologists such as Boveri (2) and Morgan (3) had recognized the existence and the influence of gradients of organic molecules on development, it was Child (4, 5) who presented the first clear demonstration of metabolic gradients and their influence on morphogenesis. Child (5) demonstrated that the regeneration of flat worms was directed by these gradients. He also discovered that both organ size and the developmental fate of tissues in embryos could be altered by experimentally induced variations in the rate of tissue metabolism (6). More recent investigations reveal that differential vascularization results in microgradient formation during development of more structurally complex organisms such as vertebrates (7, 8). The mechanism by which

0037-9727/91/1962-0117$2.00/0

metabolic gradients influence differentiation is not understood; however, several recent advances may ultimately lead to a more complete elucidation of this process. Several elements postulated to be integral to the influence of oxidative metabolism on development will be the focus of the remainder of this discussion.

Metabolic Gradients and Oxygen

The existence of metabolic gradients in developing organisms probably arises, in part, from differential supplies of nutrients, i.e., cells provided with rich nutrient supplies metabolize more rapidly than cells with poor access to nutrients. However, variations in oxygen supply imposed either by diffusion barriers or by variability of vascularization have been found to influence the products of stem cell differentiation. For example, when cultured under aerobic conditions, the syncitial slime mold *Physarum polycephalum* differentiates from ameboidal microplasmodia which have no cell walls into cells that are surrounded by cell walls. Although it survives anaerobic conditions, no differentiation is observed when cultures of the organism are placed under nitrogen; returning the cultures to an aerobic environment permits differentiation to proceed (9). Similarly, the fungus *Mucor racemosus* exists as yeast-like cells under anaerobic conditions, but aerobic conditions promote mycelial development. The rate of mammalian cell growth is strongly modulated by only slight variations in ambient oxygen tension (10). Furthermore, cultures of chick mesenchyme maintained under relatively high ambient oxygen tensions differentiate into myocytes, whereas maintenance under low ambient oxygen tensions causes them to differentiate into chondrocytes (7).

Oxygen in high concentrations is universally toxic, but the effects of hyperoxia on gene expression are probably not the result of its toxicity. For example, developing insects exhibit both oxygen-sensitive and oxygen-insensitive stages of development (11–13); this would not be expected if the effects of oxygen were mediated by a general toxicity syndrome. It is also improbable that ambient oxygen concentration influences gene expression by any direct effects exerted on aerobic metabolism. Erkell (14) induced morphologic and biochemical differentiation in neuroblastoma cells by simply culturing them under 80% oxygen for 1 week. On the other hand, he also observed that cyanide failed to inhibit differentiation induced by hyperoxic exposure. Since treatment with cyanide should effectively abolish aerobic metabolism even under hyperoxic conditions, and since cyanide alone cannot induce differentiation in neuroblastoma, it seems reasonable to infer that oxygen-mediated changes in gene expression are independent of the pathways of aerobic metabolism. However, oxygen also exerts several indirect effects on cellular metabolism. Of principle importance to this discussion is the oxygen-stimulated generation by various biochemical pathways of free radicals and other oxygen-centered oxidants collectively referred to as "active oxygen species" (AOS). The cellular generation of AOS is stimulated by increases in ambient oxygen tension and is not inhibited by the presence of cyanide (15), thus, making AOS a likely candidate for many of the effects associated with variations in oxygen concentration.

AOS

Most of the molecular oxygen consumed by aerobic cells is tetravalently reduced to H_2O; however, O_2 also readily accepts single electron transfers, and a portion of the oxygen molecules that enter cells are univalently reduced to superoxide radicals ($\cdot O_2^-$). Several cellular loci are chemically reduced sufficiently to catalyze $\cdot O_2^-$ formation, but the principle sites of generation of this radical are mitochondria, peroxisomes, and endoplasmic reticula (16–18). The half-life of $\cdot O_2^-$ is only about 1 μsec. A $\cdot O_2^-$ may either react with another $\cdot O_2^-$ or be reduced by the enzyme superoxide dismutase (SOD) to form H_2O_2. The reaction of $\cdot O_2^-$ with H_2O_2 produces the hydroxyl radical ($\cdot OH$), a highly destructive radical species that can react with practically any molecule contained in cells (17, 18).

Cells contain a variety of antioxidant defenses that limit reactions catalyzed by AOS (16, 17). Aside from SOD which removes $\cdot O_2^-$, catalase and peroxidases remove H_2O_2 (16, 18). Since enzymes are inactivated by $\cdot OH$ radicals, the presence of nonenzymic antioxidants is presumably essential to the effective removal of this radical. Cells also contain a wide variety of nonprotein antioxidants (16–18). For example α-tocopherol and β-carotene are lipid-soluble antioxidants that can limit lipid peroxidation by terminating chain reactions initiated in membrane lipids. Ascorbate and glutathione are water-soluble antioxidants that remove free radicals from the cytosol by reacting directly with them.

The number of chemical reactions thought to be catalyzed by AOS has steadily increased for several decades; yet, virtually none of these reactions may be regarded as biologically beneficial (19). Instead, it has become apparent that free radicals inactivate enzymes (20), break DNA (21), and initiate the chain reactions that peroxidize lipids (22). AOS are widely believed to play fundamental roles in cellular aging and tumorogenesis (23–25). It might be assumed that the potentially disastrous consequences of unchecked AOS reactions would effectively preclude the evolution of any cellular strategy that utilized these highly reactive substances as a biologic stimulus. However, dramatic changes in the rate of oxidant generation and in the activities of various antioxidant defenses are closely associated with the differentiation of a variety of tissues

in phylogenetically diverse organisms (10). Furthermore, experimentally induced purturbations in the cellular equilibrium between oxidant generation and removal has been repeatedly observed to alter gene expression in a number of different types of cells. These observations suggest that bursts of oxidant production may modulate gene expression (26, 27). Since fully developed organisms retain the ability to reproduce, it is also reasonable to infer that modulation of gene expression by AOS occurs in the absence of irreparable damage to DNA. Although development-associated changes have been reported for most of the known antioxidant defenses, only SOD and glutathione (GSH) are ubiquitous to aerobic cells; the presence of other antioxidant defenses is species specific. The discussion that follows examines changes in SOD activity and changes to GSH concentration that are associated with development. For a much more detailed discussion of changes reported for other antioxidant defenses see Allen and Balin (10).

SOD

As noted above, SOD (EC 1.15.1.1) is an enzyme that catalyzes removal of $\cdot O_2^-$ radicals (28). Three forms of SOD are known; they are usually classified according to the metal core of the active site of the molecule. An iron-containing SOD (Fe-SOD) is found predominantly in procaryotes. Eucaryotic cells most frequently contain a cupero-zinc (Cu/Zn-SOD) and a mangano-isozyme (Mn-SOD) (17, 18). Cell state transitions of various types are almost always associated with changes in SOD activity. Tumor cells, which have regressed from a fully differentiated state, exhibit lower SOD activity than normal cells of similar tissue origin. The loss of SOD activity associated with cellular transformation has been exhaustively reviewed (25, 29, 30) and will be discussed no further here. In contrast to changes seen during cellular transformation, SOD activity increases dramatically when cells reach terminal stages of differentiation. Changes observed during the development of various organisms are summarized in Table I.

The syncitial slime mold *P. polycephalum* provides an extremely simple model for studies on the factors that influence differentiation. When grown in shake flasks, the organism exists as small multinucleated ameboid cells referred to as microplasmodia. Microplasmodia have no cell walls, but they differentiate into clusters of hard-walled spherules (or microsclerotia) when transferred to a salts-only starvation medium (53, 54). During this transition from mitotically active microplasmodia to mitotically quiescent microsclerotia Mn-SOD activity has been observed to increase by as much as 46-fold (55). Conversely, a strain of Physarum that fails to differentiate exhibits no increase in SOD activity when maintained under identical conditions (55, 56).

The underlying cause of the development-associated increases in SOD activity is not known. Several hypotheses have been formulated to explain this phenomenon but none are entirely satisfactory. Yamanaka and Deamer (57) found that SOD activity sharply dropped when cells were trypsinized. They also observed that SOD activity was lower in tumor cells than in normal cells that are subject to contact inhibition. On the basis of these observations, Yamanaka and Deamer (57) inferred that SOD activity was cell cycle dependent and that trypsinization decreased the activity of the enzyme by stimulating mitosis. In view of the fact that many types of cells permanently cease mitosis upon reaching terminal differentiation, it is reasonable to speculate that increases in SOD associated with development may merely reflect an accumulation of nondividing cells within a tissue. However, Oberley *et al.* (58) found that an increase of Mn-SOD activity was associated with the onset of mitosis in regenerating mouse liver. Furthermore, Allen and Balin (52) found a 5-fold difference in the Mn-SOD activity of pre- and postnatal human fibroblasts that had been growth arrested. It is also noteworthy that Allen and Balin (52) employed trypsinization to remove cells from the culture vessels; however, in this study soya bean trypsin inhibitor was used to neutralize the trypsin. These observations clearly demonstrate that accumulation of SOD in cells following cessation of mitosis is not a sufficient explanation of developmental changes in SOD activity.

Several investigators have suggested that the greater oxygen content of the environment following birth stimulates the increases in SOD activity observed in neonates (39, 41, 59–61). In fact, it is now well established that SOD activity is more easily stimulated in newborn tissues than in adult tissues (39, 41, 61), indicating that only a modest change in ambient oxygen tension could stimulate a significant increase in SOD activity during development. However, this hypothesis fails for two reasons. First, organisms such as Physarum, which are not born, exhibit large increases in SOD activity during differentiation. Second, in mammalian models, SOD activity increases prior to birth (42, 43, 51, 62), yet there is no a priori reason to believe that ambient oxygen tension has been altered. Furthermore, increased SOD activity has also been observed during differentiation of cultured mammalian cells even though the ambient oxygen tension was not altered (50). Unfortunately, many investigators have reported only total SOD activity (Table I). When the different isozymes have been distinguished, it is usually changes in Mn-SOD activity alone that accounts for activity increases that accompany differentiation, while changes in Cu/Zn activity occur postnatally (Table I). Indeed,

Table I. SOD Changes Observed in Different Organisms and Tissues during Various Cell State Transitions

Organism	Tissue	Condition	% Change	Reference
Slime molds				
Didymium iridis	Whole organism	Sporulation	≈900 (units/g wet wt)	31
Physarum polycephalum[a]	Whole organism	Spherulation	4500 (units/mg protein)	32
Nematodes				
Caenorhabditis elegans[b]	Whole organism	Dauerlarvae formation	450 (units/mg protein)	33
Insects				
Ceratitis capitata[a]	Mitochondria	Pupae/adult	305 (units/mg protein)	34
Drosophila melanogaster[b]	Whole organism	First instar/adult	346 (units/μg DNA)	35
D. melanogaster[b, c]	Whole organism	Third instar/adult	447 (units/mg protein)	36
D. melanogaster[b, d]	Whole organism	Third instar/adult	−18 (units/mg protein)	36
Amphibians				
Discoglossus pictus[b]	Whole organism	Stage V/Stage XIV	57 (units/mg protein)	37
Rana ridibunda[b]	Whole organism	Stage V/Stage XIV	200 (units/mg protein)	37
	Whole organism	Stage III/Stage XIV	1500 (units/mg protein)	37
Xenopus laevis[e]	Oocytes	Oogenesis	≈900 (units/oocyte)	38
Mammals				
Rabbit[b]	Lung	Neonate/adult	50 (units/mg protein)	39
—[b]	Lung	Fetal/neonate	110 (units/mg DNA)	40
—[b]	Lung	Fetal/adult	390 (units/g wet wt)	41
—[b]	Erythrocytes	Bone marrow maturation	151 (units/mg protein)	42
Rat[b]	Lung	Neonate/adult	103 (units/g wet wt)	41
—[b]	Lung	Neonate/adult	294 (units/mg DNA)	41
—[b]	Lung	Neonate/adult	29 (units/mg protein)	39
—[e]	Lung	Fetus/adult	≈1500 (units/mg protein)	43
—[b]	Lung	Neonate/adult	52 (units/mg protein)	44
—[b]	Lung	Fetus/adult	88 (units/g wet wt)	45
—[b]	Lung	Neonate/adult	150 (units/mg protein)	46
—[b]	Lung	Neonate/adult	200 (units/mg DNA)	46
—[a]	Lung	Fetus/neonate	≈375 (units/unit cytochrome oxidase)	43
—[e]	Hepatocytes	Neonate/adult	335 (μg/mg protein)	47
—[e]	Liver	Neonate/adult	90 (units/mg protein)	48
—[a]	Liver mitochondria	Fetus/adult	560 (units/mg protein)	49
—[b]	Brain	Neonate/adult	0 (units/mg protein)	48
—[b]	Brown adipose	Neonate/adult	0 (units/mg protein)	48
—[e]	Brain	Neonate/adult	85 (μg/mg protein)	47
—[a]	Brain	Neonate/adult	≈4500 (μg/mg protein)	47

Table I. Continued

Organism	Tissue	Condition	% Change	Reference
Mouse[b]	Lung	Neonate/adult	23 (units/mg protein)	39
Hamster[b]	Lung	Neonate/adult	28 (units/mg protein)	39
Guinea pig[b]	Lung	Neonate/adult	66 (units/mg protein)	39
Human[b]	Monocytes	*In vitro* differentiation	250 (units/mg protein)	50
—[b]	Lung	Neonate/adult	67 (units/g wet wt)	41
—[b]	Lung	Neonate/adult	547 (units/mg DNA)	41
—[b]	Placenta	4 weeks/40 weeks	490 (units/g wet wt)	51
—[e]	Skin fibroblasts	Adult versus gestational age <17 weeks	0 (units/mg protein)	52
—[a]	Skin fibroblasts	Adult versus gestational age <17 weeks	≈500 (units/mg protein)	52

[a] Mn-SOD activity.
[b] Total SOD activity.
[c] Males.
[d] Females.
[e] CuZn SOD activity.

only the Cu/Zn-SOD isozyme is sensitive to ambient oxygen concentration in neonatal mammals (43).

Finally, it has been postulated that developmental increases in SOD activity occur because they are stimulated by increases in the rate of free radical generation (26, 27). It would seem noteworthy that free radical generators such as the herbicide paraquat stimulate SOD activity in the nondifferentiating strain of Physarum (55). This may suggest that the failure of the nondifferentiating strain to exhibit increased SOD activity in differentiation medium was due to an absence of any stimulus. In addition to increased SOD activity, various parameters of cellular oxidation also increase during differentiation. H_2O_2 concentration increases in Physarum during spherulation (56) and in human monocytes during differentiation *in vitro* (50). Lipid peroxidation (56, 63) and cyanide-resistant respiration are both stimulated during spherulation in Physarum. None of these changes are observed in the nondifferentiating strain maintained under identical culture conditions (55). Lipid unsaturation and therefore susceptibility to peroxidation is greatly elevated in the tissues of newborn mammals (45, 49, 64–67); however, whether lipid peroxidation is elevated prior to birth is presently unclear. Presently, there is sufficient evidence to support the hypothesis that, at least in some tissues, SOD activity increases during development because it is stimulated by an upsurge in the rate of cellular $\cdot O_2^-$ generation.

Glutathione and Sulfhydryl Status

GSH is a tripeptide consisting of glycine, cysteine, and glutamic acid moieties. The–SH group of the cysteine moiety can react with oxidants and neutralizes them. The concentration of GSH in cells is enormous; it frequently exceeds concentrations of 1 m*M*. The redox state of cells is expressed as the ratio of the concentration of oxidizing equivalents to the concentration of reducing equivalents (16, 68, 69). Because it is the most prevalent constituent in the cellular pool of reducing equivalents, even modest variations in GSH concentration can strongly modulate redox state. Thus, aside from its antioxidant properties, GSH acts as a cellular redox buffer (69, 70). A very tight control of redox state is necessary for the maintenance of protein charge and function, and also contributes to the maintenance of cellular polarity and the compartmentalization of ionic charges within cells. Additionally, GSH is a co-factor for a number of enzymes (16, 70).

Cells from a variety of organisms respond to an oxidative challenge by increasing their concentration of GSH. For example, in houseflies, GSH concentration becomes proportional to ambient oxygen concentration within 3 hr of placing the insects under different atmospheric O_2 partial pressures. Similar effects are seen in cultured human fibroblasts after 24 hr. Other forms of oxidative challenge also induce GSH synthesis; insects and slime molds both exhibit a higher GSH concentration when exposed to paraquat (55, 71) and mammalian liver and gastric tissues increase GSH concentration in response to chemically induced stress (72, 73). Because large changes in GSH concentration can be induced by relatively small increments in the level of oxidative stress, the existence of metabolic gradients in embryos might be expected to stimulate the forma-

tion of redox gradients. Indeed, GSH gradients have been observed in housefly larva (70), and sulfhydryl gradients have been found in the developing roots of carrots (74).

Terminal differentiation is frequently associated with large shifts in redox equilibrium in organisms as diverse as slime molds (75), carrots (76), the Norway spruce (77), insects (70), and mammalian tissues (61, 78). Cells that lose mitotic capacity tend to become more oxidized during the final phases of differentiation. As much as 80% of the GSH content of Physarum is lost during spherulation. Unfortunately, in Physarum, as in other types of cultured cells, it is difficult to assess the extent of GSH oxidation because membranes are permeable to oxidized glutathine (GSSG) and permit it to pass freely from the cell into the surrounding medium (75). The loss of GSH in Physarum is at least partly due to a decline in γ-glutamylcysteine synthetase, the rate controlling step of GSH synthesis (10). GSH concentration declines by roughly 60% during metamorphosis in insects (70). Similarly, the level of GSH decreases from 24 nmol/mg protein in rat fetal lung to around 12 nmol/mg protein in the lung tissue of newborns (61).

Tissues which retain a high regenerative capacity, such as mammalian liver, exhibit no loss of GSH during their differentiation; however, the ability of these tissues to resynthesize GSH following depletion progressively diminishes as development proceeds (10). GSH concentration increases during the development of meiotic tissues. For example, a 3-fold increase in GSH is observed in rat testes between 8 and 29 days of age (79). Cellular transformation is often associated with large increases in GSH concentration (24, 80). Melanomas have been reported to contain up to 10 times the GSH content of normal human skin (81). Similarly, regression from a fully differentiated state, such as during regeneration, is associated with large sustained increases in tissue sulfhydryl levels; during the subsequent redifferentiation phase sulfhydryl levels decline. Studies performed in this laboratory reveal that changes in GSH metabolism associated with cellular transformation produce patterns of cellular response to oxidative stress that are uncharacteristic of either fetal or adult tissues. Both fetal and adult human lung fibroblasts exhibit a GSH concentration that is directly proportional to ambient oxygen tension. Conversely, transformed fibroblasts exhibit a nonlinear GSH response to oxidative stress. The response of GSH is altered even when the transformed cells are derived from fetal tissue (Fig. 1). Other tumor lines examined (including pigmented and unpigmented melanoma and squamous cell carcinoma) change GSH concentration in response to oxidative stress in a manner similar to SV-40 transformed WI-38 depicted in Figure 1.

The increase of GSH synthesis associated with

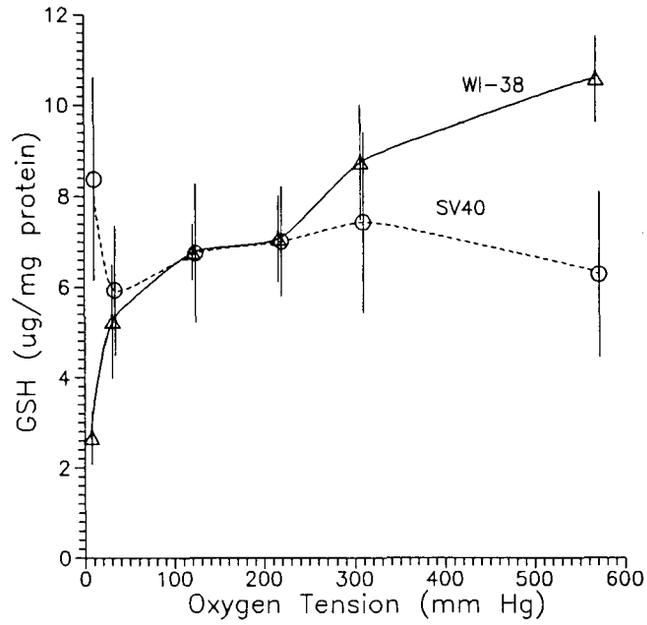

Figure 1. Glutathione concentration in human fetal lung fibroblasts (WI-38) and SV40-transformed WI-38 cultured at different oxygen partial pressures. The cells were grown for 96 hr at 5% oxygen and were then cultured for an additional 48 hr at one of six different oxygen tensions. Cells were harvested by trypsinization; the trypsin was neutralized with soya bean trypsin inhibitor.

regeneration and cellular transformation is probably due to increased mitotic activity. There is a considerable body of evidence which implicates GSH as an important factor that governs the onset of mitosis (80, 82). It is also probable that the decrease in GSH concentration that accompanies terminal differentiation is partly due to changes in the rate of GSH synthesis associated with the cessation of cell cycle. As in the case of development-associated changes in SOD activity, the decrease in GSH concentration that occurs during terminal differentiation cannot be explained solely on the basis of a reduced mitotic frequency. In the whole body homogenates of developing houseflies, GSSG accumulates as GSH declines, indicating that oxidation accounts for a portion of the loss of GSH that accompanies differentiation (Fig. 2). Cultures of differentiating carrot cells exhibit a significant loss of GSH accompanied by an increase in GSSG (76). The differentiation of 3T3 L1 preadipocytes also occurs with a concomitant decline in GSH concentration and a sharp rise is GSSG (78). No loss of GSH is observed during the differentiation of mouse erythrocytes, which might be expected since the mitochondria of these cells atrophy during differentiation and thus eliminate a principle source of AOS generation (10, 83).

Oxidant and Antioxidant Interactions Play a Causal Role in Differentiation

In the above discussion evidence has been presented to demonstrate the existence of metabolic gra-

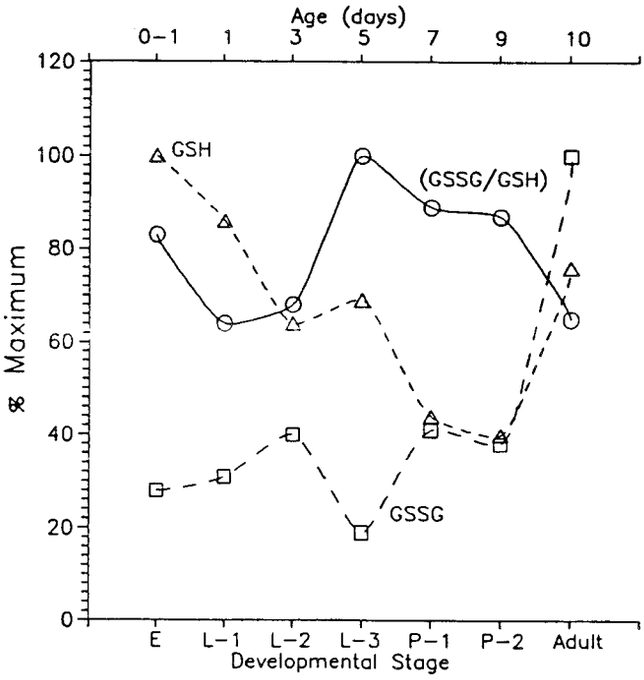

Figure 2. Relative changes in GSH, GSSG; and the ratio of GSSG to GSH during the development of the housefly. E, egg; L1, first instar; L2, second instar; L3, third instar; P-1, early pupae; P-2, late pupae; and 1-day-old adult.

dients in embryos. Regional variations in metabolic rate, which forms the basis of metabolic gradients, will presumably establish corresponding regional variations redox status and in the rate of free radical generation and oxidant production, i.e., redox gradients and AOS gradients. Changes in the SOD activity and cellular redox status known to occur during differentiation have also been presented. Yet, whether the existence of metabolic gradients or changes in antioxidant defense levels are induced by advancing developmental programs, or are causal to the process of differentiation has only recently been explored.

As discussed above, Physarum exhibits a striking increase in Mn-SOD activity during spherulation. No increase in SOD activity is observed in a nondifferentiating strain cultured under identical conditions. Treatment of the nondifferentiating strain with bovine Cu/Zn-SOD encapsulation in liposomes enhanced the SOD activity of the organism 4.5-fold and stimulated its differentiation into viable spherules (32). Similarly, liposomally encapsulated SOD has been employed to induce differentiation in Friend erythroleukemia (84). The SOD mimetic Cu(II)(3,5-diisopropylsalicylate) has been employed to induce differentiation in neuroblastoma (25).

Antioxidants such as tocopherol have also been reported to inhibit tumor cell growth (85–87). Biomimetic SOD has been employed to retard tumor growth apparently by inhibiting promotion (88). Studies conducted in this laboratory reveal that treatment of cultured melanoma and squamous cell carcinoma lines with suspensions of tocopherol, β-carotene, or SOD-containing liposomes halts their growth. Furthermore, treatment of the unpigmented melanoma line with SOD-containing liposomes induced the appearance of dark granules similar to pigment granules; however, the exact composition of these granules could not be determined. Neither tocopherol nor β-carotene appeared to induce the formation of these cytoplasmic granules, possibly indicating differences in the mechanism of effects exerted by these compounds (unpublished data). Paradoxically, antioxidants other than SOD inhibit spherulation of differentiating strains of Physarum and also retard the rate of differentiation of Friend cells treated with hexamethylene bisacetamide (32, 84, 89). Oxidants such as cumene hydroperoxide and liposomally encapsulated xanthine oxidase induced differentiation in the nondifferentiating strain of Physarum and in Friend cells. Oxidants also accelerate spherulation in differentiating strains of Physarum (55). Indeed, treatment with SOD containing liposomes stimulates the level of H_2O_2 in Physarum. These observations lead to the rather puzzling conclusion that SOD stimulates differentiation by causing cellular oxidation (10, 32). Supportive of this hypothesis is the observation that overproduction of SOD in HeLa cells, by an inserted SOD gene, has also been reported to stimulate lipid peroxidation (90).

Although it is an antioxidant enzyme, Cu/Zn-SOD can catalyze $\cdot OH$ radical formation from H_2O_2, the product of the enzymes dismutation of $\cdot O_2^-$ (91). This suggests that the increase in oxidation observed during the spherulation of differentiating strains of Physarum and during induction of spherulation with SOD-containing liposomes might arise from the direct catalysis of oxidation reactions by the SOD molecule. However, Mn-SOD cannot catalyze $\cdot OH$ radical formation (91), even though the increase in Mn-SOD activity that occurs during normal spherulation is strongly correlated with an increase in several parameters of oxidation (56). Furthermore, liposomally encapsulated bacterial Mn-SOD can induce differentiation in the nondifferentiating strain of Physarum (unpublished observation). A second mechanism that might explain the increased cellular oxidation associated with increased SOD activity is that some oxidation reactions are reversibly inhibited by $\cdot O_2^-$ (92–94). Enzymes that generate free radical by-products are also protected from autoinactivation by SOD (95, 96). The possible involvement of other mechanisms should not be ruled out; albeit, other possible mechanisms are presently unknown. What is apparent from the existing evidence is that increases in SOD activity associated with development are partly due to induction of the enzyme, and it is the oxidation associated with the increase in SOD

rather than the antioxidant properties of the enzyme that seem to play a role in stimulating changes in gene expression (10).

Changes in the redox status of developing tissues modulate changes in gene expression. The sulfhydryl status of the pathogenic fungus *Histoplasma capsulatum* regulates the transition of the organism from a filamentous form (found in soil) to its infectious yeast phase (97). Sulfhydryl-containing compounds have been observed to modulate the expression of heat shock genes in chick embryo cells (98), sulfhydryl groups are the target sites of several different inducers of heat shock genes in eukaryotic cells (99, 100). Furthermore, sulfydryl oxidation with diamide induces the synthesis of heat shock proteins (101). Inhibition of GSH synthesis accelerates spherulation in Physarum (75). Conversely, addition of GSH to cultures or stimulation of GSH synthesis inhibits differentiation in Physarum (75, 89). The postnodal pieces of chick embryos, which normally exhibit no inductive capacity, acquire the ability to stimulate neural induction when soaked in a solution of GSH or cysteine (102). The normal pattern of differentiation in postnodal pieces can be altered by soaking the pieces in GSH of cysteine. Following this treatment the postnodal pieces of chick embryos acquired the ability to form several different tissues (103). This suggests that chemically reducing these tissues can alter cellular commitment to differentiate into a specific tissue. Interestingly, higher concentrations of sulfhydryl compounds inhibit RNA synthesis (103).

Mechanisms of Oxidative Influence on Gene Expression

Four discrete mechanisms by which AOS alter gene expression have been identified. They are (i) AOS stimulation of changes in cellular ion distribution, (ii) direct interactions with chromatin-controlling proteins, (iii) oxidative influences on the cytoskeleton and nuclear matrix affect chromatin configuration and premessenger RNA processing, and (iv) changes in redox state caused by AOS make the cellular environment permissive to preexisting, but inactive factors, A brief discussion of each of these follows.

Changes in Ion Distribution. Cells maintain an extremely tight control over free ion concentration. Because ionic concentration affects a very large number of biochemical pathways, a sustained increase in the concentration of free ions could rapidly lead to cellular chaos and death. Cells maintain their ion concentration at very low levels. The mitochondria and endoplasmic reticula are the primary sites for storage of ions within cells. Stimulation of various biochemical pathways by ions appears to proceed by release of ions from cellular stores followed by a very rapid sequestration of the ions (104).

Ionic sequestration by cells is strongly modulated by their redox state (105). It has been postulated that an upsurge in the rate of free radical generation during development could stimulate a release of mitochondrial stores of ions, particularly calcium ions, and thereby initiate a cascade of biochemical events that lead to changes in gene expression (10). A direct demonstration of Ca^{2+} release during differentiation has recently been presented by Nations *et al.* (63). They found that microplasmodia of Physarum transferred to a salts-only starvation medium exhibit a formation of electron-dense granules in their mitochondria during the first 6 hr. Electron probe analysis revealed the composition of these granules to be calcium and phosphorus. Concomitant with the accumulation of calcium in mitochondria is an initial decrease in Mn-SOD activity and an early increase in NADH concentration. After 6 hr these mitochondrial granules begin to slowly disappear, being virtually gone by 30 hr of culture when differentiation is about 90% completed. It is during this same period that SOD activity increases and NADH levels fall by more than 85% (63). The loss of GSH will, of course, make the cellular environment more prooxidizing and may also permit free radicals to escape reduction, but it does not directly stimulate Ca^{2+} release from mitochondria (105). The fact that GSH can at least indirectly modulate cellular sequestration of calcium has been clearly demonstrated in cultured mammalian cells (106, 107). On the other hand, in Physarum, the GSH concentration decreases as calcium granules are accumulating in mitochondria during the initial 6 hr of culture of differentiation medium (63). It is probable that the loss of NADH and the increased AOS generation account for the Ca^{2+} release observed as spherulation proceeds (63, 105).

Direct Activation by Oxidants. Treatment of bacteria with small amounts of H_2O_2 induces the synthesis of more than 30 proteins. Nine of these are now known to be under the control of the *oxy R* gene. The gene is particularly interesting because it also seems to regulate its own transcription (108). A second intriguing point is that activation of the protein is not due to disulfide bond formation, conjugation with short chain sulfhydryl compounds such as GSH, or reduction of a metal ion core. Storz *et al.* (108) have postulated that the *oxy R* protein may have an active site that captures oxidants directly with a concomitant conformational change in the protein. The conformational change releases the protein from the *oxy R* gene and stimulates its binding to the other genes that it regulates. The mechanism by which the *oxy R* protein stimulates transcription of other genes is presently unknown. A similar observation has been made in cultured human fibroblasts (109). Oxidation stimulated by ascorbic acid (probably by a redox cycling mechanism) has been observed to stimulate transcription of the collagen gene. Antioxidants such as α-tocopherol inhibit the transcription of colla-

gen associated with peroxidation. It has been postulated that a direct interaction of lipid peroxidation by-products with a transactivating protein alters its binding properties to activate collagen transcription or that direct interaction of the collagen gene and peroxidation by-products make it more accessible to transcription factors (109).

Cytoskeleton, Nuclear Matrix, and RNA Processing. A physical factor that strongly modulates gene expression is the spatial arrangement of chromatin; i.e., chromatin configuration (110–113). The redox status as well as the ionic milieu of cells affects chromatin configuration (114–116). Changes in chromatin configuration arise partly from direct interactions with ions (115, 117). Thus, a change in redox status that stimulates the release of cellular stores of ions may indirectly alter chromatin configuration and gene activity.

It has been very clearly demonstrated that oxidants induce chromosomal puff formation in a variety of organisms (98–101). The formation of chromosomal puffs is at least partly due to changes in the cellular electrolytic balance (118, 119); however, oxidants also influence nuclear matrix proteins and the karyoskeleton (120). The karyoskeleton is thought to play an important role in RNA processing by providing anchor points for pre-mRNA (121–123). Although anchoring pre-mRNA appears to be important to processing, its precise role is unknown. It has been demonstrated that disruption of the karyoskeleton by $\cdot O_2^-$ stimulates the release of unprocessed RNA from nuclei (124). It would seem reasonable to postulate that, at physiologic steady-state levels, $\cdot O_2^-$ regulates, or at least influences, the anchoring of pre-mRNA to the karyoskeleton. The cytoskeleton reciprocally transmits information from the cell membrane and chromatin (125). Variations in membrane fluidity and membrane charge or intracellular ion balance induced by free radicals can therefore alter chromatin configuration (125–127). It would seem noteworthy that ionic gradients are established at relatively early stages of development (104, 128), and as discussed above redox gradients also exist in embryos. The influence of redox and ionic gradients on chromatin configuration and RNA processing alone could account for the positional divergence of cellular developmental fates.

Environmental Changes Permissive to Gene Expression are Modulated by Redox Status. The activity of proteins is strongly influenced by their charge. Protein charge is largely a product of microenvironmental factors such as pH, redox state, and surrounding ions (129). One recently discovered example of environmental influences on gene expression is the mechanism of transcriptional control of ferritin synthesis. When the cellular concentration of free iron is low, ferritin translation is suppressed; an increase in free iron stimulates the translation of ferritin by as much as 50- to 100-fold (130, 131). A 28-nucleotide sequence at the 5′ end of the ferritin called the iron-responsive element is the binding site for iron-sensitive translational repressor or iron-responsive element-binding protein (130, 131). The ability of the iron-responsive element binding protein to bind to the iron-responsive element is dependent upon the sulfhydryl status of the protein. An environment which is highly reduced favors binding, whereas a prooxidizing environment inhibits binding (132). It is important to note that no change in the concentration of regulatory components is necessary to alter translation. In this case it is the change in binding affinity stimulated by a corresponding change in redox state that regulates expression of the ferritin gene.

A second interesting example was the recent observation that modulation of GSH concentration could be employed to control cell cycle in human T lymphocytes (133). This result was inferred to indicate that GSH was essential for steps regulating DNA synthesis. A partial confirmation of this hypothesis can be derived from a study by Roederer et al. (134). They found that even the expression of human immunodeficiency virus could be controlled by modulation of the cellular GSH concentration (134).

Changes in redox state associated with development tend to be extremely large and are usually permanent (see discussion above). It seems probable that development-associated changes in redox status make the microenvironment permissive to changes in gene expression either by altering the activity of translational controls or the activity of chromatin-controlling proteins. Although the evidence to support the existence of a general mechanism of this type is limited, the evidence that does exist is compelling. The extent to which this or the other three mechanisms discussed above influence development has not been determined; however, the number of studies conducted to determine AOS effects on gene expression appear to be increasing, and it seems probable that a more complete elucidation of any AOS role in development is imminent.

Conclusions

Metabolic gradients are established at very early stages of development; they arise because of interactions between physical constants such as diffusion and the thermodynamic restraints of energy transfer in cells and biologic parameters such as the number of catabolic enzymes and membrane receptors present in the cells in any given region of an embryo. As metabolic gradients become established, they may alter the cellular environment by concomitantly precipitating the formation of redox gradients and AOS generation gradients. Both AOS and redox status can influence transcriptional and translational controls of gene expression, and both affect the activity of the protein products

of the genes. This discussion began with the mention of the control of development by homeotic genes. The considerable lapse between the time when homeotic genes are expressed and the time when they begin to exert an influence on development indicates that their affect on the expression of other genes, itself, may be initiated by permissive changes in the cellular environment. By this reasoning, changes in gene expression can occur with relatively little effect until the microenvironment is altered to permit full activity of new gene products. Although it has been possible to demonstrate the existence of redox and AOS influence on development and on the expression of specific genes, there is presently no conclusive evidence that links these physical factors with homeotic genes. Nevertheless, it is evident that if the effects described here are a part of a general mechanism, its implications will be far-reaching in studies of development, aging, and cellular transformation.

This work was supported in part by the generosity of the Pew Trusts. I thank D. Martin Carter, MD, PhD, for suggestions helpful in the completion of this manuscript.

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