Lipid Rafts and Keratinocyte Apoptosis: Regulation *via* Death Receptors and Akt

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Abstract: Lipid rafts are cholesterol-rich plasma membrane domains that regulate signal transduction. Lipid rafts can be disrupted by perturbing cholesterol metabolism, either by inhibition of cholesterol synthesis (e.g. by statins), by cholesterol sequestration or removal from the membrane (e.g. by methyl-β-cyclodextrin or filipin III) or by substitution with cholesterol derivatives, which do not support raft assembly (e.g. 5-cholestene 5-β-ol). Work performed in our laboratories as well as by others documented that lipid raft disruption precipitates classic apoptosis in normal and transformed keratinocytes and inhibited cell proliferation. Two mechanisms seem to play a particularly important role. One is mediated *via* the membrane death receptors such as Fas and TRAIL. Cholesterol depletion causes a ligand-independent activation of these receptors and activation of caspases. The other is mediated *via* the survival kinase Akt. Raft disruption causes a rapid inhibition of Akt *via* dephospphorylation of the regulatory sites Thr^{308} and Ser^{473}. The mechanism of this was the abrogation the binding of Akt and the major Akt kinase, PDK-1 to the membrane *via* pleckstrin homology (PH) domains. Diminished Akt activity results in deactivation of mTOR, activation of FoxO3a and an increased sensitivity to apoptotic stimuli. Thus, the integrity of lipid rafts is required for the activity of Akt and cell survival and may serve as a potential pharmacological target in the treatment of epidermal cancers.

The plasma membrane contains nanometer-large dynamic microdomains enriched in cholesterol, sphingolipids and gangliosides called "lipid rafts". These structures are important regulators of signal transduction, membrane geometry, and lateral movement of the molecules (see refs. [1-5] and elsewhere in this volume).

Since lipid rafts form spontaneously only in the narrow range of cholesterol concentration, the perturbation of membrane cholesterol disrupts lipid raft integrity and has been used extensively to study their role in signaling pathways. Of note, many receptors and enzymes, crucially involved in the regulation of cell survival and apoptosis have been reported to associate with rafts. For example, the epidermal growth factor receptor (EGFR), which proper function is a prerequisite for keratinocyte survival, can be transiently activated by cholesterol depletion due to increased ligand-independent dimerization and cross-phosphorylation [2, 6] after release from the rafts. On the other hand, cholesterol depletion induces the activity of the stress kinase p38 [7] which signals towards cell cycle stop and increased differentiation of keratinocytes.

Old biochemical observations documented that cancer tissues contain more cholesterol than healthy tissue and it has been speculated that this may have functional significance for tumor growth [8]. Increased cholesterol content in the tissue and patients' sera have been found in actinic keratoses [9, 10]. Interestingly, cholesterol depletion from the membranes of leukemic cells and various other cancer cells resulted in apoptosis and increased sensitivity to chemotherapeutic drugs [11]. In this paper we review evidence that the same may hold true for normal and transformed human keratinocytes.

DEPLETION OF MEMBRANE CHOLESTEROL CAUSES KERATINOCYTE APOPTOSIS

Our laboratories have been the first to report that depletion of cholesterol in cultured keratinocytes causes apoptosis [12, 13]. These observations have been independently confirmed [14]. Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with sensitivity to apoptosis induced by cholesterol-depleting agents [14]. Apoptotic keratinocytes in cholesterol-depleted cultures show the typical features of programmed cell death, including caspase activation, DNA fragmentation, nuclear shrinkage and formation of apoptotic bodies. Apoptosis has been induced by a variety of cholesterol-modifying agents such as methyl-β-cyclodextrin, statins, ceramides (which displace cholesterol from the rafts), filipin III (an agent that inactivates cholesterol in the membrane by complex formation) or cholesterol oxidase. However, a more detailed electron microscopic analysis of cholesterol-depleted cells revealed other interesting features, including a striking swelling of mitochondria, loss of mitochondrial potential $\Delta\Psi_m$ and autophagocytosis[1] It is thus possible that

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autophagocytic cell death is also involved in the cell death due to lipid raft disruption. This issue is currently investigated in our laboratory.

CHOLESTEROL DEPLETION CAUSES A LIGAND-INDEPENDENT ACTIVATION OF DEATH RECEPTORS IN KERATINOCYTES

Activation of membrane death receptors such as Fas or TRAIL constitutes one of the two major pathways leading to apoptosis. Under most circumstances, these receptors are activated upon binding of the cognate ligands, such as FasL (Fas ligand). There is extensive evidence that association with or dissociation from lipid rafts modulates the activatory state of death receptors [15]. In most cells, the activation of Fas takes place within the milieu of lipid rafts and raft disruption blocks Fas-dependent apoptosis [16, 17]

A theory has been forwarded suggesting that Fas, upon recruitment to rafts, forms large complexes with other components of the apoptotic machinery, including caspase-8 and is further activated intracellularly precipitating apoptosis [18]. However, in some cell types, including epidermal keratinocytes and squamous cell carcinoma the opposite is true: depletion of cholesterol causes Fas clustering outside the rafts and activation of cell death pathway [13, 19]. The mechanism seems to be similar: upon cholesterol depletion large membranous aggregates of Fas are observed which co-localize with caspase 8 and FADD (Fas-associated death domain protein). It seems that this activation is not dependent of the presence of ligand. Moreover, keratinocytes, which are slightly cholesterol depleted, are more sensitive to the death inducing ligands, such as FasL and TRAIL. Since carcinoma cells contain more cholesterol that normal cells, it is in principle possible to utilize this phenomenon for tumor sensitization to death ligands thus minimizing their systemic toxicities.

AKT/PROTEIN KINASE B IS RESPONSIVE TO LIPID RAFT DISRUPTION AND MEDIATES KERATINOCYTE APOPTOSIS

It is important to note that activation of surface death receptors is neither necessary nor sufficient for keratinocyte apoptosis after cholesterol depletion. Fas-induced apoptosis can normally be blocked by caspase-8 inhibitors, but cholesterol-depleted keratinocytes undergo apoptosis even in the presence of this inhibitor (unpublished observations).

Akt/protein kinase B is a cardinal regulator of cell survival and its pathologic increase in activity causes carcinogenesis [20, 21]. Membrane receptors including the ErbB family of surface receptors, IGFRs or Ras stimulate Akt kinase activity *via* the phosphoinositide 3-kinase (PI3K) [22, 23]. PI3K catalyzes the conversion of membrane phosphatidylinositol 4-5-bisphosphate PIP2 to phosphatidylinositol 3,4,5-trisphosphate PIP3, to which both Akt and its immediate activator phosphatidylinositol-dependent kinase 1 (PDK-1) bind *via* pleckstrin-homology (PH) domains [24, 25]. Full activation of Akt requires phosphorylation of Thr^{308} by PDK-1, followed by a second phosphorylation at Ser^{473} [26]. Downstream components of the Akt-PI3K signaling cascade include mammalian target of rapamycin (mTOR)/ $p70^{S6K}$ pathway [27, 28] and the

apoptosis-related Forkhead familiy of transcription factors FoxO, causing their emigration from the nucleus [29, 30].

Lipid rafts are known to be enriched in PIP2 and PIP3 [31] and are therefore likely to provide the particularly permissive milieu for the interaction between PDK-1 and Akt. Indeed, Akt resides in the detergent-resistant (raft-like) membrane fraction. Using the fluorescence correlation spectroscopy technique Lasserre *et al.* [32] demonstrated that recruitment of Akt to lipid rafts facilitates its enzymatic activation. Guo *et al.* [33] confirmed these results using yet another technique of fluorescence resonance energy transfer with an artificially expressed Akt activity reporter. They showed a higher Akt activity with faster activation kinetics within lipid rafts and impaired Akt signaling in cholesterol-depleted cells. However, the activation of Akt in rafts seems to depend on both the cell type and the stimulus involved. In NIH 3T3 cells used by Guo *et al.* [33] platelet-derived growth factor (PDGF) stimulated Akt in both raft and non-raft regions, albeit more efficiently in the raft domains. In contrast, insulin-like growth factor 1 (IGF-1) stimulates Akt that is localized in raft regions and in this case raft disruption by e.g. cholesterol depletion abolishes the total cellular Akt activity to a higher degree.

In keratinocytes and keratinocyte-derived cancer cell lines the activity of Akt is also dependent on the integrity of lipid rafts. Li *et al.* [14] showed that in A431 cells cholesterol depletion by methyl-β-cyclodextrin or simvastatin caused apoptosis accompanied by Akt deactivation. Confirming our previous observations [2] they showed that At deactivation occurs in spite of the fact that EGFR is activated. Our recent results[2] confirm these findings. In both normal keratinocytes and keratinocte cell lines (HaCaT, A431) methyl-β-cyclodextrin and other cholesterol-active agents such as filipin III, cholesterol oxidase, simvastatin and 5-cholesten-5-β-ol induced an abrupt dephosphorylation of Akt at its regulatory sites (Thr^{308} and Ser^{473}), inactivation of mTOR and nuclear export of FoxO3a. The mechanism of Akt deactivation in keratinocytes is reduction of the number of the phosphoinositide-enriched sites in the membrane to which Akt and PDK-1 bind *via* PH domains. Thus, in keratinocytes lipid rafts can function as reaction nanochambers allowing close proximity and enzymatic interaction between PDK-1 and Akt. Cholesterol perturbation causes disruption or dispersion of lipid rafts and movement of the growth factor-PI3K complexes into the more fluid, non-raft areas of the membrane. As a consequence, the PIP3 produced by this enzyme is dispersed in the membrane instead of being concentrated in the highly organized raft nanodomains. As a consequence, the binding of PH domain proteins, such as Akt, PLCδ and PDK-1 to the membrane can be impaired which precludes their spatial interaction and Akt phosphorylation.

The discovery of the cholesterol-dependence of Akt in keratinocytes is likely to have practical consequences. Akt in keratinocytes constitutes a dominant survival pathway and its blockade leads to apoptotic and autophagic cell death,

even in the presence of an active EGFR-ERK axis. Decreased Akt activity renders the cells more susceptible to chemotherapeutics. The PI3K-Akt-mTOR pathway is hyperactivated in many types of cancer providing survival advantage to transformed cells [34] often due to inactivatory mutations in the gene coding PTEN lipid phosphatase (phosphatase and tensin homologue deleted on chromosome ten) mediating dephosphorylation of PIP3 to PIP2. Since cancer cells are enriched in caveolae and lipid rafts comparing to normal cells [14], pharmacologic cholesterol depletion may be a promising method for increasing chemosensitivity to the cytostatics in patients with malignant tumors. Preliminary observations on high dose statins support this notion [11].

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