

Dean's Lecture

Molecular Signaling Regulating Anchorage-Independent Growth of Cancer Cells

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Abstract

Normal adhering cells undergo apoptosis shortly after loss of adhesion to substratum, a phenomenon known as "anoikis." *In-vitro*-transformed cells and cancer-derived cells are able to survive and grow in the absence of anchorage to the extracellular matrix (ECM) and their neighboring cells. This represents one of the most important oncogenic properties of cancer cells. Integrin-ECM-mediated function is essential for survival and growth of normal adhering cells, while cancer cells are able to abrogate this requirement. This article will review and summarize the recent findings from our laboratory about the molecular signaling pathways important for the regulation of anchorage-independent survival and the growth of transformed fibroblasts and epithelial cells. Our study has shown that integrin-ECM-mediated signaling and cytoskeletal architecture play an essential role in effective recognition of the substrates by activated protein-tyrosine kinases (PTK) and their subsequent signaling functions. Among the various oncogenic PTK-activated pathways, phosphatidylinositol 3-kinase (PI3K)/Akt signaling is the most critical for anchorage-independent survival and growth. The activation of signal transducer and activator of transcription-3 (Stat3) and its function overlap partially with that of the PI3K/Akt in promoting anchorage-independent growth. Among the Rho family guanosine triphosphatases (GTPase), Cdc42, and to some extent Rac1, also appear to be important for promoting anchorage-independent growth.

Key Words: Anoikis, PI3 kinase, Stat3, Rho GTPases, anchorage.

Glossary

Akt: oncogene identified in the Akt-8 murine retrovirus isolation from an AKR/J mouse thymoma

Bad: a member of the bcl family apoptosis regulatory protein

ca: constitutively activated

ca PI3K: an activated PI3 kinase mutant

Cdk: cyclin-dependent kinase

CEF: chicken embryo fibroblasts

dn: dominant negative

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: EGF receptor

FAK: focal adhesion kinase

FKHRL1: forkhead-related transcription factor L1

GDP: guanosine diphosphate

GSK: glycogen synthase kinase

GTPase: guanosine triphosphate

IGF-1: insulin-like growth factor-1

IGFR: IGF-1 receptor

ILK: integrin-linked kinase

IR: insulin receptor

JAK: Janus kinase

MAPK: mitogen-activated protein kinase

MC: methylcellulose

PI3K: phosphatidylinositol 3-kinase

PLC γ : phospholipase C γ

PTK: protein-tyrosine kinase

RIE: rat intestinal epithelial

RPTK: receptor protein tyrosine kinase

Src: sarcoma-inducing gene in Rous sarcoma virus

STAT: signal transducers and activators of transcription

TGF: transforming growth factor

Introduction

ONE OF THE HALLMARK PROPERTIES of *in-vitro*-transformed cells and cancer cells is that they are capable of anchorage-independent growth. Normal cells require synergistic signaling func-

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tions elicited from growth factor-growth factor receptor interaction as well as from interaction between cell surface molecules and extracellular matrix (ECM), for survival and cell cycle progression (1). Complicated crosstalk and regulation takes place between the function of a growth factor receptor and integrin-ECM-mediated signaling (2–4). The important mitogenic signaling including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and Rho family guanosine triphosphatases (GTPase) are known to be regulated by integrin-ECM signaling functions (2–7). Two integrin-associated protein kinases, focal adhesion kinase (FAK) and integrin-linked kinase (ILK), have been reported to play an important role in the process of crosstalk (8–10). Upon deprivation of substratum adhesion, normal fibroblasts undergo growth arrest, whereas normal epithelial cells undergo apoptosis, a phenomenon called “anoikis” (1, 11–13). The mechanism for the trigger of apoptosis in response to deprivation of cell adhesion to ECM is not clear, but it is likely to involve both the mitochondria and death-receptor-mediated activation of apoptotic caspases (11–13). There has been evidence to suggest that release of proapoptotic factors such as Bim and Bmf from cytoskeletal sequestration may play a role in the initial stage of the apoptotic process (14, 15).

Transformed cells and cancer cells have reduced requirements for substratum adhesion and are able to survive and grow under the nonadhesive or anchorage-independent condition by inhibiting anoikis-related apoptotic pathways (11–13; Fig. 1). This *in vitro* property of cells correlates very well with their *in vivo* oncogenic potential. Therefore, one convenient assay of the potential of anchorage-independent growth, namely colony-forming ability in semisolid agar medium, becomes a reliable assessment of the oncogenic capability of cells. But what is the advantage for cancer cells of attaining such capability in the process of oncogenesis? One of the two most outstanding properties of cancer cells is uncontrolled growth and metastasis; the latter, in particular, defines the advanced stage and high malignancy of cancers. The metastatic process requires cancer cells to detach and migrate away from the primary tumor and to intravasate into blood or lymphatic vessels. The cells need to survive the anchorage-independent condition during the process, including their transportation through the circulation system until reaching a distant target organ, where they extravasate and begin to form initial metastasis. The migratory, invasive and anoikis-resistant capabilities of cancer cells endow them with the metastatic potential. Inhibiting these capabilities of cancer cells would effectively prevent the

Anchorage Independence of Transformed Cells

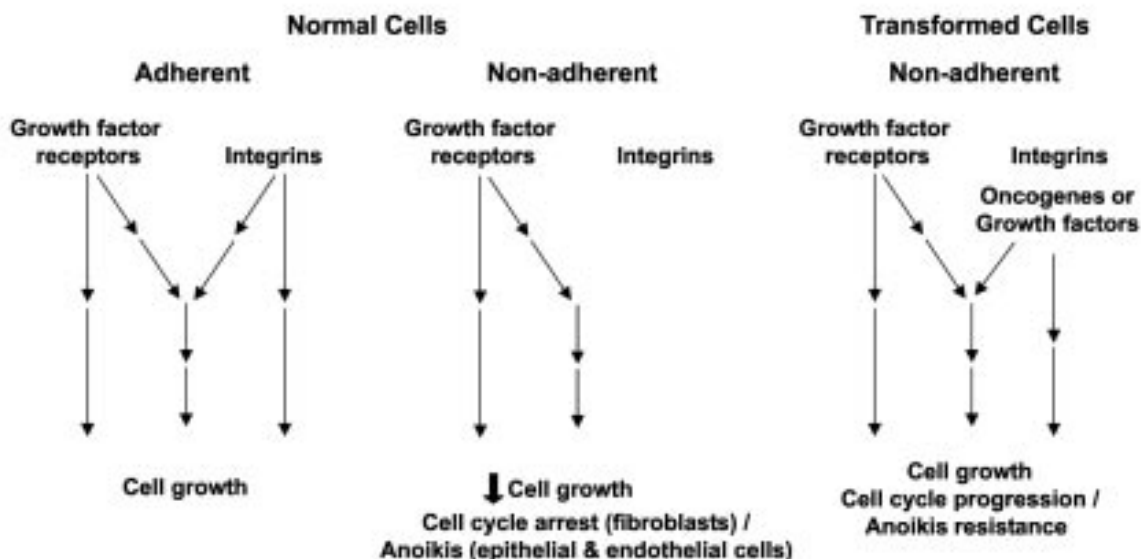


Figure. Anchorage independence of transformed cells.

process of metastasis formation. However, the molecular basis underlying these characteristics of cancer cells is not fully understood.

Do cancer cells constitutively activate certain signaling pathways to abrogate the requirement for integrin-ECM-mediated signaling function for survival and growth? Do those activated signaling pathways block the apoptosis process resulting from deprivation of adhesion and at the same time promote the cell cycle progression? What are the most critical signaling function(s) capable of sustaining survival and growth of cells under anchorage-independent conditions? This article will review and summarize some of the findings from our laboratory using certain activated receptor protein tyrosine kinase (RPTK) oncogenes in the transformation of fibroblasts and epithelial cells. Our results will be compared with those in the literature.

Anchorage-Independent Phosphorylation and Activation of PTK Substrates and Signaling Molecules

Normal protein-tyrosine kinases (PTK) initiate their signaling function upon cognate ligand binding in the case of receptor PTKs, or physiological activation in the case of non-receptor PTKs, by autophosphorylation and phosphorylation of their immediate substrates and the signaling molecules. Oncogenic or activated PTKs essentially recognize and activate the same set of substrates and signaling molecules as their normal counterpart PTKs except at a higher intensity and constitutively. To assess the effect of ECM interaction and cytoskeletal architecture on substrate recognition by PTKs, we have adopted an anchorage-independent culturing condition by growing cells in methylcellulose (MC)-containing medium (16). Cells grown in the MC medium suspension can be readily recovered for protein analysis (16). Chicken embryo fibroblasts (CEF) expressing v-Ros, v-Src and v-Yes oncogenic PTKs were grown in monolayer or in the MC medium for 48 hours, and then compared for total cellular protein tyrosine phosphorylation by these oncogenic PTKs. The v-Ros is an oncogenic receptor PTK, whereas v-Src and v-Yes are two oncogenic nonreceptor PTKs; all have been derived from avian sarcoma retroviruses (17–19). The results revealed that, overall, tyrosine phosphorylation of cellular substrates was greatly reduced in cells grown in the MC medium compared to those grown in monolayer, despite the fact that the respective PTKs

were equally abundant and active in cells grown in either adhering or nonadhering condition (16). All the mitogenic signaling pathways, including those of MAPK, phospholipase C γ (PLC γ), PI3K, Stat3, E-cadherin and integrin/FAK were dramatically attenuated in nonadhering cells. However, PI3K and Stat3 remained significantly activated in v-Ros-transformed CEF when compared to control or transformation-defective v-Ros mutant expressing cells, suggesting that PI3K and Stat3 signaling may be important for v-Ros-induced, anchorage-independent growth (see below). A similar phenomenon was observed in rat intestinal epithelial (RIE) cells expressing an oncogenic insulin-like growth factor-1 (IGF-1) receptor or stimulated with epidermal growth factor (EGF) and grown under the adhering or nonadhering conditions (S. Uttam Singh and L.-H. Wang, unpublished). Addition of ECM molecules such as fibronectin, laminin, collagens or Matrigel (Sigma, St. Louis, Missouri) to the semisolid medium did not correct the inability of those activated PTKs to recognize and activate their substrates and downstream signaling molecules when cells are grown under the nonadhering condition. These results indicate that cell-ECM interaction and spatial architecture of the cytoskeleton are critical for PTKs to be able to effectively interact and phosphorylate their substrates and initiate the signaling functions. This is true not only in normal cells, but also in the transformed cells expressing constitutively activated PTKs. Our results are consistent with the well-established link between integrin signaling and activation of Ras-Raf-MAPK pathway (3, 4, 9, 20). Integrin-ECM-mediated signaling is able to amplify and prolong the growth factor receptors-mediated mitogenic signaling. Thus, integrin/ECM signaling and cytoskeletal structure appear to play a critical role in properly positioning the substrate molecules for their recognition and activation by PTKs and in subsequent augmenting of the signaling process.

Role of PI3 Kinase/Akt Signaling in Anchorage-Independent Growth

The PI3K pathway can be activated by different mechanisms, mainly via growth factor receptors and integrin/ECM-mediated signaling, and it plays an important role in a multitude of cellular functions, including cell growth, survival, migration and invasion (21–24). Using the NIH3T3, CEF and RIE cells as well as the MC medium culturing system, we examined the role of PI3K/Akt (see

glossary) signaling in anchorage-independent growth. Our results showed that the oncogenic insulin receptor (IR) and IGF-1 receptor (IGFR)-transformed RIE cells were inhibited by the PI3 kinase inhibitor LY 294002 and the p70S6 kinase inhibitor rapamycin for their ability to form colonies in semisolid agar medium (25). By contrast, Src-transformed (see glossary) RIE cells were relatively resistant to the similar inhibitors for colony formation, suggesting that Src apparently was able to activate certain pathway(s) with a function redundant to that of PI3K/Akt for anchorage-independent survival. Similarly, v-Ros-transformed CEF or activated EGFR-Ros-chimera-expressing NIH3T3 cells were highly sensitive to the inhibitors of PI3K and p70S6 kinase for colony formation (26). Inhibition of the colony-forming ability by pharmacological inhibitors of the PI3K-signaling molecules was confirmed by transfection and expression of the dominant negative (dn) mutants of PI3K and Akt in those transformed cells (26). Conversely, activated PI3K was able to rescue the colony-promoting activity of two transformation-defective mutants of v-Ros (26). The colony-forming ability of NIH3T3, CEF and RIE cells expressing those activated PTKs, by contrast, was relatively resistant to inhibitors of the MAP kinase (25, 26). Interestingly, Vav3, the third member of the Vav family Rho GTPases nucleotide exchange factors, upon its activation, was able to promote the evasion of contact inhibition (i.e., focus formation) in NIH3T3 cells efficiently, but was unable to promote their anchorage-independent growth in soft agar (27, 28). Upon screening of a number of activated molecules involved in mitogenic signaling functions, an activated PI3 kinase mutant (caPI3K) was found to be able to cooperate with the activated Vav3 for promoting colony formation of the NIH3T3 cells, while neither activated Vav3 nor caPI3K alone was able to do so (28). It would be interesting to identify which signaling function of Vav3 is involved in such collaboration. Our observations are consistent with those in the literature demonstrating the importance of PI3K activation and signaling in various oncogene-transformed cells and in cancer-derived cells (29–35).

How does PI3K signaling promote the anchorage-independent growth? The effect of activated PI3K on anchorage-independent expression and activation of cells-cycle-regulatory proteins was investigated. While expression of various cyclins and cyclin-dependent kinases (Cdk) was not affected in v-Ros-expressing CEF under nonadhering conditions, the cyclin-A-associated

Cdk activity appeared to be augmented in the v-Ros-expressing CEF, but not in the control or v-Ros mutant-expressing cells (16). The increase in cyclin-A-associated Cdk activity was PI3K dependent, since it was blocked by the PI3K inhibitor. The PI3K-dependent increase of Cdk2 activity was important for v-Ros-induced colony formation, since inhibition of the Cdk2 activity to the level of control CEF blocked the colony-forming ability of v-Ros-expressing cells (16).

In another study, we showed that EGF and transforming growth factor (TGF)- β 1 synergistically protect RIE cells from anoikis, which was also blocked by PI3K inhibitors (S. Uttamsingh and L.-H. Wang, unpublished). Activation of MAPK and Akt was prolonged in the EGF- and TGF- β 1-treated cells grown under nonadhering conditions, leading to phosphorylation and inactivation of pro-apoptotic proteins such as glycogen synthase kinase (GSK)-3 β and forkhead-related transcription factor L1 (FKHRL1) as well as decreased level of cyclin inhibitor p27^{KIP1} and p16^{INK4a} (S. Uttamsingh, KT Nguyen and L-H Wang, unpublished). Taken together, PI3K signaling appears to be able to block the apoptotic process and enhance the anchorage-independent cell cycle progression at the same time. Akt was previously shown to phosphorylate directly or indirectly and inactivate pro-apoptotic proteins such as caspase 9, Bad (see glossary) and FKHRL1 in different cell systems (36–43). Our results are consistent with those findings. Overall, the activated PI3 kinase signaling plays a critical role in protecting cells from anoikis by inactivating certain key apoptotic molecules and at the same time enhancing anchorage-independent cell cycle progression by inhibiting the cyclin inhibitors and enhancing certain Cdk activity.

The Role of STAT Signaling in Anchorage-Independent Growth

Among the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling components, Stat3 stands out for its involvement in regulating cell growth and transformation as opposed to cell maturation, cell stress and acute phase responses for most of the other STAT signaling functions (44–46). Stat3 was initially found to be activated in Src-transformed cells (47). Subsequently, activation and signaling of Stat3 was shown to be important for cell transformation by several oncogenes (48–51). Moreover, constitutively activated Stat3 alone was demonstrated to have a considerable degree of transforming and tumorigenic

potential (52). A variety of naturally occurring cancer cells have been shown to harbor an elevated level of Stat3 or an elevated level of activated Stat3 (53–59). Those observations firmly place Stat3 on the list of pro-oncogenic molecules. However, its precise role in oncogenesis in general and in anchorage-independent growth in particular remains unclear. We showed that activation of Stat3 was required for EGF receptor (EGFR)-Ros- and oncogenic IGF-1R-induced anchorage-independent growth of NIH3T3 cells (50). As mentioned above, activation of Stat3 and PI3K stand out as providing two enhanced signaling pathways in v-Ros-expressing cells maintained under the nonadhering conditions. Both signaling pathways were shown to be important for v-Ros-mediated anchorage-independent growth. The dn Stat3 and dn PI3K additively inhibited v-Ros-induced colony formation of CEF. Conversely, the ca Stat3 and ca PI3K additively rescued the colony-promoting activity of two transformation-defective mutants of v-Ros. The ca Stat3 was able to partially rescue the inhibition of EGFR-Ros-induced colony formation of NIH3T3 cells by PI3K inhibitor LY294002 (26). Those observations suggest that the Stat3 signaling function partially overlaps with that of PI3 kinase in promoting anchorage-independent growth. The mechanism by which Stat3 signaling actually leads to promoting anchorage-independent growth is not clear. However, it was shown that activation of Stat3 was accompanied by the upregulation of cyclin D1, c-myc and Bclx (58), whose changes are consistent with the protection of cells from anoikis and promotion of anchorage-independent cell cycle progression. Nevertheless, direct evidence for those molecules in mediating Stat3-induced colony formation and cell transformation awaits further study.

Role of the Rho Family GTPases in Anchorage-Independent Growth

All members of the small GTP-binding GTPases superfamily exist in cells as either the inactive guanosine diphosphate (GDP)-bound or the active GTP-bound form, the latter of which is regulated positively by nucleotide exchange factors and negatively by GTPase-activating proteins (60, 61). The most well-studied members of the Rho family GTPases are RhoA, Rac1 and Cdc42, which were initially known for their function in regulating the formation of stress fibers, lamellipodia and filopodia, respectively (62–65). Subsequently, they were

shown to be involved in other cellular functions as well, notably cell growth, motility and transformation (62–71). While Rho GTPases function to regulate cytoskeleton, they are themselves regulated by integrin signaling (72–75). Using the same cell and oncogene systems described above, we have examined the roles of Rho, Rac and Cdc42 in cell transformation including anchorage-independent growth. Using an oncogenic IGFR and its loss-of-function mutants, we analyzed the roles of Rho, Rac and Cdc42 in this oncogenic IGFR-induced transformation of NIH3T3 cells (69). Our results indicated that those GTPases played distinct roles in the oncogenic IGFR-induced evasion of contact inhibition (assayed by focus formation) vs. anchorage-independent growth (assayed by colony formation). Dominant negative (dn) Rho most potently inhibited focus formation of the cells, whereas dn Cdc42 inhibited colony formation most effectively among the three GTPases. Conversely, constitutively activated (ca) Rho rescued the focus-forming ability of the IGFR mutants, while ca Cdc42 rescued their colony-forming ability most efficiently. Thus, Rho signaling appears to play a more important role for evasion of contact inhibition, whereas Cdc42 signaling is more important in promoting anchorage-independent growth. This conclusion about the role of Cdc42 in anchorage-independent growth is consistent with the reports that Cdc42 mediated integrin signaling for activation of PI3 kinase and Akt (76, 77). The observation of the importance of Rho for focus formation was supported by one of our other studies, where we observed that Vav3-induced focus formation of NIH3T3 cells was most potently inhibited by dn Rho (28). Cdc42 most likely promotes anchorage-independent growth via activation of Rac, PI3K and Akt. The mechanism for Rho-mediated evasion of contact inhibition remains an intriguing open question.

Conclusion

Integrin/ECM signaling plays a critical role for growth and survival of normal adhering cells. Deprivation of anchorage destines cells to undergo growth arrest and apoptosis. Cancer cells are able to survive the lack of anchorage, and thus the lack of integrin signaling, by preventing the apoptosis process. This occurs because of the intrinsic constitutive activation of certain signaling pathway(s), notably those of the PI3K, Stat3 and GTPases, discussed above. While activation of mitogenic and pro-survival

signaling can logically explain the anchorage-independent growth of cancer cells, it is quite plausible that mutation and inactivation of tumor suppressors and pro-apoptotic factors in cancer cells could endow them with similar oncogenic ability. This possibility is not discussed here. Other signaling functions, such as Ras/Raf/MAPK and PKC pathways, which are likewise not discussed here, may also help promote anchorage-independent growth. Nevertheless, the PI3 kinase/Akt is apparently the converging point and is the key signaling function for survival. To date, we have not observed a single oncogene transformed or a cancer-derived cell line that is able to undergo anchorage-independent growth when PI3K/Akt function is blocked, with the exception of Src-transformed RIE cells (mentioned above), which are partially resistant to the inhibition of PI3 kinase (25). This could be explained in part by the fact that Src is a potent activator of Stat3 (47) and our evidence has suggested that Stat3 signaling is able to compensate partially for the lack of PI3K/Akt function for anchorage-independent growth (26, see above). Therefore, both Stat3 and PI3K/Akt, especially the latter, are important and prudent molecular targets for anti-cancer intervention.

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