

## CLUSTERING OF SPECIFIC MOLECULES IN SHED VESICLES.

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In several tumor cell lines serum addition causes release of vesicles that bud from the cell surface and can be purified from cell conditioned media. These vesicles are known to be involved in cell migration and tumor progression.

We recently demonstrated that FGF-2, a growth factor devoid of the classical signaling sequence, is secreted as a component of these vesicles. In order to analyze how molecules are clustered in shed vesicles we followed their intracellular movements by immunofluorescence techniques. The role of cytoskeletal components was analyzed using molecules such as paclitaxol, nocodazole, colchicin and cytochalasin which destabilize their organization. In the absence of serum, no clear localization of FGF-2 was observed. After serum addition, FGF-2 was localized partially in the nucleus and nucleolus, and partially in granules near the plasma membrane. Nocodazole and paclitaxol, which interfere with microtubular organization, inhibit FGF-2 nuclear localization but do not appear to modify FGF-2 movements toward the plasma membrane. Cytochalasine, which interferes with actin polymerization, decreases FGF-2 clustering in granules localized near the cell membrane. In summary, microtubular organization seems to be required for FGF-2 nuclear localization while actin filaments appear to be needed for FGF-2 translocation toward the plasma membrane.

In a different set of experiments, we analyzed localization of neutral ceramidase (ncDase) and of Sphingosine Kinase (SphK). Ceramidase catalyzes ceramide hydrolysis giving rise to sphingosine, which in turn can be phosphorylated to S1P by SphK. S1P is an important signaling molecule involved in induction of cell migration and apoptosis. SphK-1 was known to be shed into the extracellular medium by an unconventional mechanism, we hypothesized that shed vesicles could vehicle its release.

We therefore analyzed the localization of membrane-bound isoforms of ceramidase (ncDase) and of SphK (SphK-1 and SphK-2) by western blotting and Immunofluorescence techniques. Immunolocalization showed that ncDase is located into the plasma membrane and in cellular extensions. The concentration of ncDase was found to be higher in extracts of shed vesicles than in cell extracts. SphK-1 was found to be localized in plasma membrane and in vesicles, which appear to be enriched in this enzyme. SphK-2 was preferentially located in the nucleus and it was not detected in vesicles. In conclusion, ncDase and SphK were found to be clustered in shed vesicles. In order to analyze the role of SphK-1, either in the shedding phenomenon or in vesicle functions, we used transiently transfected SK-Hep1 cells, which overexpress SphK or express a non-functional mutant of this enzyme.